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Transgenic cardiac-targeted overexpression of human thymidylate kinase

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Thymidylate kinase (TMPK) is a nucleoside monophosphate kinase that catalyzes phosphorylation of thymidine monophosphate to thymidine diphosphate. TMPK also mediates phosphorylation of monophosphates of thymidine nucleoside analog (NA) prodrugs on the pathway to their active triphosphate antiviral or antitumor moieties. Novel transgenic mice (TG) expressing human (h) TMPK were genetically engineered using the α-myosin heavy chain promoter to drive its cardiac-targeted overexpression. In '2 by 2' protocols, TMPK TGs and wild-type (WT) littermates were treated with the NA zidovudine (a deoxythymidine analog, 3′-azido-3′deoxythymidine (AZT)) or vehicle for 35 days. Alternatively, TGs and WTs were treated with a deoxycytidine NA (racivir, RCV) or vehicle. Changes in mitochondrial DNA (mtDNA) abundance and mitochondrial ultrastructure were defined quantitatively by real-time PCR and transmission electron microscopy, respectively. Cardiac performance was determined echocardiographically. Results showed TMPK TGs treated with either AZT or RCV exhibited decreased cardiac mtDNA abundance. Cardiac ultrastructural changes were seen only with AZT. AZT-treated TGs exhibited increased left ventricle (LV) mass. In contrast, LV mass in RCV-treated TGs and WTs remained unchanged. In all cohorts, LV end-diastolic dimension remained unchanged. This novel cardiac-targeted overexpression of hTMPK helps define the role of TMPK in mitochondrial toxicity of antiretrovirals.
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Mitochondria are unique cellular organelles with their own DNA (mitochondrial DNA, mtDNA), replication machinery (polymerase gamma, pol γ),[1] and mitochondrial deoxynucleotide triphosphate (dNTP) pools essential for the synthesis of mtDNA.[2] mtDNA synthesis occurs throughout the life of the cell, independent of nuclear DNA (nDNA) synthesis and is essential for mitochondrial energy production. Cardiomyocytes (terminally differentiated, non-replicating cells) require constant high-energy phosphates to sustain muscular contractions. Disrupted mtDNA replication can lead to left ventricle (LV) hypertrophy, cardiomegaly, and organ dysfunction.

The importance of maintaining the dNTP precursor pools is underscored by human genetic diseases in which mutations in enzymes for dNTP synthesis adversely affect the rate and/or fidelity of mtDNA replication.[3–5] Mitochondrial deoxythymidine triphosphate (dTTP) may be generated by two separate pathways in mammalian cells. *De novo* synthesis is accomplished from ribonucleotides in the cytosol and is followed by import into the mitochondria.[6] Alternatively, deoxythymidine (dThd) is imported into the mitochondria and three sequential kinase-mediated phosphorylations generate dTTP. Cycling cells largely depend on the first pathway (*de novo* synthesis), whereas resting cells use the alternative pathway mediated by three mitochondrial kinases.[7] Intramitochondrially, the first phosphorylation is mediated by the mitochondrial thymidine kinase (TK2),[8] which we have studied earlier.[9,10] The second phosphorylation is mediated by the cytosolic thymidylate kinase (TMPK).[11] In this study, we targeted cytoplasmic

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overexpression of TMPK. We hypothesized that in these TMPK transgenic mice (TGs), thymidine diphosphate (TDP) precursors in the cytoplasm would accumulate as the myocytes are quiescent. These precursors ultimately would be delivered into the mitochondria through specific nucleotide transporters (eg SLC25[12]) where they could potentially impact mtDNA replication.

Nucleoside analogs (NA) are well-recognized anticancer and antiviral agents.[13] The thymidine NA zidovudine (3′-azido-3′deoxythymidine, AZT) is a *bona fide* treatment for HIV/AIDS. NAs are 'prodrugs' whose therapeutic activity relates to conversion to the active triphosphated *N* (eg, AZTTP).[11,14,15] Like their native counterparts, NAs are phosphorylated serially into TPs mediated by the same kinases (eg, TK2 and TMPK). For AZT, the rate-limiting step is the conversion of AZTMP to AZTDP by TMPK.

As treatment with AZT is associated with mitochondrial toxicity to tissues, a novel *in vivo* model using overexpression of TMPK was engineered to delineate the role of TMPK in phosphorylation of native nucleosides and NAs. Using '2 by 2' protocols, TGs and wild types (WTs) were treated by daily gavage with each nucleoside analog solubolized in appropriate vehicles (AZT in carboxymethylcellulose (CMC) or racivir (RCV) in saline), and compared to respective vehicle controls. Experiments here characterize a novel cardiac-targeted TMPK TG and highlight the role of TMPK in mtDNA replication, cardiac function, and how TMPK determines toxicity of NAs.

MATERIALS AND METHODS

Generation of Alpha-Myosin Heavy Chain Promoter-Driven hTMPK TG Mice

Established methods[16] were used as described earlier.[17] They applied to the hTMPK cDNA construct.[18] The TMPK gene included the R200A mutation. This mutation was initially done to facilitate structural studies on TMPK, and it does not affect the kinetic behavior of the enzyme.[19] Three transgenic lines (A, B, C) were established for the targeted overexpression of hTMPK. FVB/n (JAX stock, Jackson Lab, Bar Harbor, ME, USA) was the inbred background. Animals bred true for five generations (to ensure germline transgenesis) before beginning experimental studies. No gross phenotype was recognized in TGs or WT littermates. No changes in behavior, growth, maturation, breeding behavior, or Mendelian distribution of TG occurred.

TG Gene Copy Analysis

To determine the relative copy number in each line, the level of hTMPK was analyzed semi-quantitatively from murine tail DNA extracts using real-time PCR and Light Cycler TaqMan Master kit. Target genes were amplified using specific primers for hTMPK (forward TMPK/LC: 5′-ATGAGAACGGGGCTTTCC-3′, reverse TMPK/LCR: 5′-TTTGGAAGCATCCACCATCT-3′, and Universal Probe Library probe #31; Roche Diagnostics Corp., Indianapolis, IN, USA) and a 'housekeeping' gene, GAPDH (forward: 5′-GATGCTACAAGCAGGCCTTT-3′, reverse: 5′-GCAGAAAGC AAGGGCAAA-3′, and Universal ProbeLibrary probe #4; Roche Diagnostics Corp.). DNA amplification was performed using LightCycler 480 (Roche Diagnostics Corp.) on individual tissues extracted from at least five to seven mice within each line. Relative copy number dosage was normalized to endogenous murine TMPK (single copy gene) from WT.

Further verification/selection for the hTMPK was accomplished using PCR amplification of tail DNA using two specific primers that identify part of the α-MHC promoter in tandem with the hTMPK gene, thereby avoiding amplification of the endogenous murine TMPK (TMPK foward 5′-CACATAGAAGCCTAGCCCACA-3′ and TMPK reverse 5′-TATAGTCGACTCACTTCCATAGCTCCCACAGCGG-3′).

Antiretroviral Treatment Protocols

All procedures complied with Emory Institutional Animal Care and Use Committee and NIH guidelines. WT and TG littermates (both genders) were age-matched (8–12 weeks) at the start of treatment protocols. Food and water were provided *ad libitum* in a 12 h light/dark, humidity and temperature controlled environment at Emory. Antiretroviral drugs were from the manufacturers (compliments of Emory Center for AIDS Research Pharmacology Core). Dosing was by daily gavage (morning). Treatment regimens resembled those used in human therapy and included AZT (0.22 mg/day; 0.25 ml in 1% CMC), RCV (1.025 mg/day in saline), or respective vehicles alone. At day 35, animal weights and echocardiographic measurements were made[20] and animals were terminated. This termination date was chosen experimentally based on our previous data that show measurable changes in cardiac function occur within 35 days of treatment with AZT.[9,10,21–23] Heart samples were retrieved rapidly and frozen (−80°C storage) for subsequent DNA extraction and analysis, or were fixed for histology (10% neutral buffered formalin; Fisher Scientific, Pittsburgh, PA, USA) and electron microscopy (EM).

Echocardiography of TG Mice

LV mass and LV end-diastolic dimension (LVEDD) were determined as described earlier using echocardiography (ECHO) in age- and gender-matched (littermate) WT and TGs.[23] Briefly, LV mass was calculated from LV wall thickness and dimension measurements using an established formula.

mtDNA and nDNA Quantification in Heart Tissue Using Real-Time PCR

Methods used were based on modifications of those used by others[24] and detailed by us.[9,25] Amplification was performed in a LightCycler 480 (Roche Diagnostics). Efficiency curves corresponding to mitochondrial and nDNA were used to determine the ratio of mtDNA to nDNA in each sample. For mtDNA abundance, the resultant values were expressed as

mean ± standard error (s.e.m.), normalized to vehicle-treated WT mean (set at 1.0). A value of $P<0.05$ was considered statistically significant.

Fine Structure Pathological Evaluations of Mitochondrial Changes with Transmission EM of Mitochondrial Damage in hTMPK TGs

Mitochondrial ultrastructure was evaluated using transmission EM to support data from ECHO and mtDNA abundance using methods that resemble those used regularly in the laboratory.[26] Each EM photograph was reviewed independently by two investigators for the presence of structurally abnormal mitochondria, increased numbers of mitochondrial profiles per field, intramitochondrial lamellar bodies, abnormal cristae density, cristae reduplication, mitochondrial swelling, and intramitochondrial paracrystals as performed by others.[27]

Histological Analysis

Heart samples were processed, sectioned (6 μm), stained with hematoxylin and eosin (H&E), and examined microscopically as done by us earlier.[28] Images were stored electronically to compare histopathological features.

Experimental Analysis and Statistics

Values for LV mass, LVEDD, and mtDNA were compared in WT, TGs, and NA-treated cohorts using ANOVA in GraphPad Prism 4 (GraphPad, San Diego, CA, USA). *Post hoc* testing used Newman–Keul's and unpaired *t*-test. A value of $P<0.05$ (determined by Student's unpaired *t*-test) was considered statistically significant.

RESULTS

hTMPK Cardiac-Targeted Transgenic Design

The mutant human TMPK clone as pGEX-RB plasmid was used.[18] hTMPK was directionally inserted into a plasmid construct containing the alpha-myosin heavy chain promoter (α-MyHC)[16] using the nucleoside restriction sites *Sal*I and *Hind*III (Figure 1a). This α-MyHC-hTMPK construct targeted overexpression of hTMPK in cardiac myocytes, based on primary activation of the α-MyHC promoter activity exclusively in cardiomyocytes. Primers designed to amplify the region overlapping α-MyHC promoter and *hTMPK* gene (~750 bp) provided a way to identify the transgene unambiguously while preventing amplification of the endogenous murine TMPK gene. Dot blot imaging was obtained routinely on DNA isolated from tail clippings for F1–F5 progeny. Signal for positive TGs was compared to WT progeny, TMPK positive (plasmid DNA, dark dots) and negative controls (no plasmid, white dots) as indicated (Figure 1b). Semi-quantitation of hTMPK copy number was accomplished from the signals on the dotblots (eg, row A: positions 3–4, 6–7, and 9, high copy) *versus* other positive dot blots (eg, row B: positions 1–2, 5–8, medium copy). Alternatively, all subsequent generations of progeny were routinely identified using PCR amplification with positive TGs identified by the production of a ~750 bp band (Figure 1c).

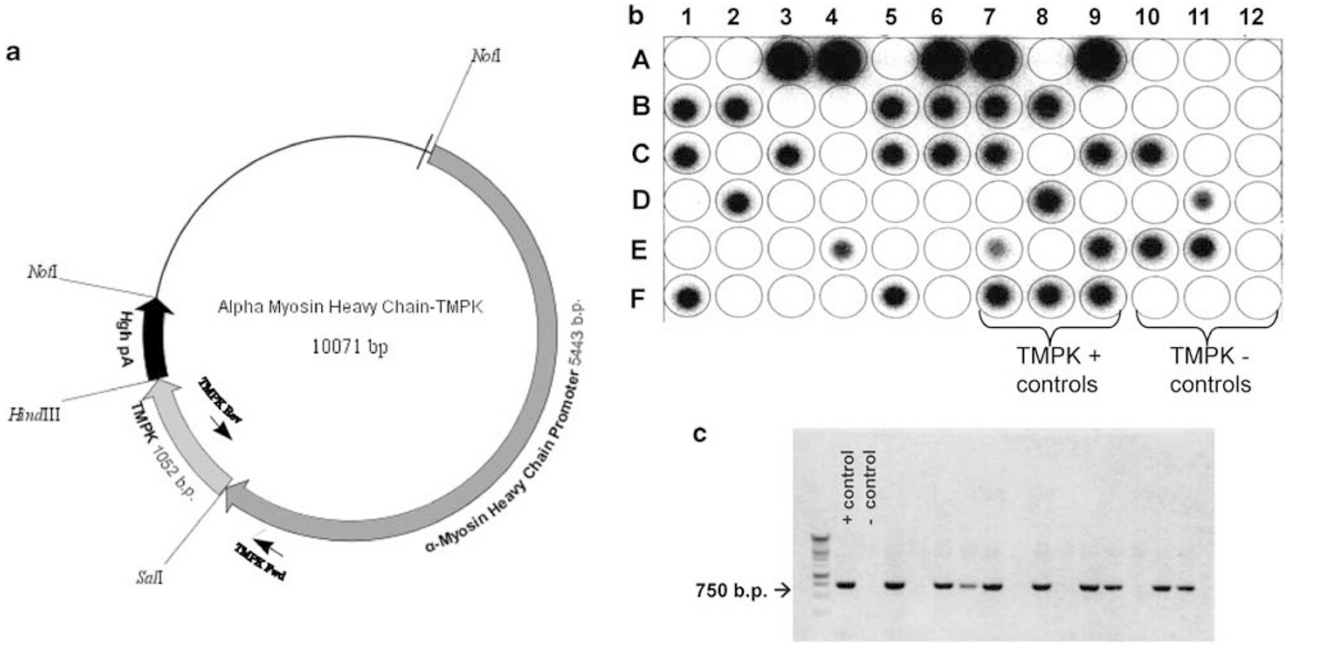

Figure 1 Generation and verification of TMPK transgenesis. (**a**) Molecular map summarizes α-MyHC-TMPK construct with restriction sites and primer-binding sites for positive identification of transgenes. Representative dot blot (**b**) and agarose gel image of PCR amplification products (**c**) show selection of positive TGs from tail DNA of individual progeny from TG line(s) derived.

Murine TG Characteristics

Cardiac-targeted transgenic overexpression of hTMPK was accomplished in three lines (operationally labeled as line A, B, and C). Animals bred true for five generations. In general, no gross phenotypic changes occurred in TGs or FVB/n WTs. No changes in behavior, growth, maturation, breeding behavior, or Mendelian distribution of TGs were found in any of the three TG lines.

The relative hTMPK gene copy number in the lines was semi-quantitatively determined (Table 1). All demonstrated multiple copy numbers of hTMPK. Lines A and B exhibited ~5-fold in excess of WT (FVB/n, single murine copy) whereas TG C line exhibited 25-fold excess. Line C was selected for these initial pharmacological studies based on its high copy number. It was reasoned that functional impact of TMPK TG on mtDNA biogenesis and cardiac function with or without an NRTI (NA) treatment would be most notable in a high copy number line.

Table 1 hTMPK TG gene copy number

Line	TMPK gene dosage[a]	Copy number
A	5	Medium
B	5	Medium
C	25	High
FVB/n WT	1	Single copy

[a]Relative TMPK copy number normalized to FVB/n WT (single copy gene).

mtDNA Abundance

Age-matched cohorts of TGs and WTs were treated with AZT (CMC vehicle), RCV (saline vehicle), or the respective vehicle control for 35 days. At study termination, mice were killed and extracts of total DNA were isolated from cardiac tissues from individual mice for each cohort. Both mtDNA and nDNA steady-state levels were determined using real-time PCR and resultant mtDNA/nDNA ratios (mtDNA abundance) were calculated and normalized as mean ± s.e.m. Vehicle-treated TMPK TGs exhibited a small decrease (although not statistically significant, $P>0.05$) in mtDNA abundance compared to vehicle-treated WTs (Figure 2a and b).

Likewise, AZT had only a moderate effect on mtDNA abundance in WTs (Figure 2a). This effect can be attributed to the endogenous activity of mouse TMPK. Mouse TMPK is more active than its human counterpart with AZTMP.[29] Therefore, in mice more AZT-triphosphate is generated relative to the condition in human cells. It is this activation of AZT by endogenous mouse TMPK that moderately decreases the mtDNA-abundance. In the case of RCV treated WT animals, no decrease in mtDNA is observed (Figure 2b).

In contrast, AZT-treated TGs exhibited a significant decrease in cardiac mtDNA abundance ($P<0.001$ and <0.01, respectively) (Figure 2a), suggesting disruption of mitochondrial biogenesis. With RCV treatment, TMPK TGs exhibited a significant decrease in mtDNA abundance only when compared to WTs (Figure 2b).

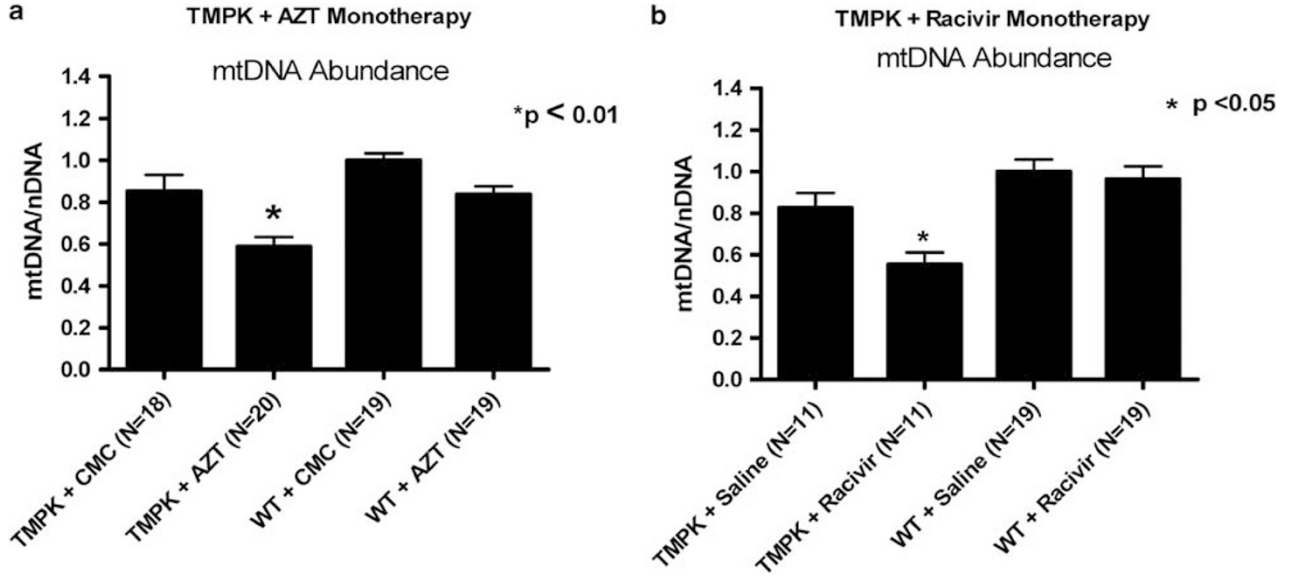

Figure 2 Cardiac mtDNA abundance in TMPK TGs and WTs after NRTI treatment. Total DNA was extracted from cardiac tissues isolated from TMPK TG and WT cohorts treated with zidovudine (AZT in CMC vehicle), racivir (RCV in saline vehicle), or vehicle alone for 35 days. mtDNA abundance was assessed using a ratio of mtDNA/nDNA as determined by real-time PCR amplification. Cardiac mtDNA abundance was significantly decreased in TMPK TGs treated with AZT (**a**) or RCV (**b**) compared to WT littermates.

Ultrastructural (EM) Features of the Mitochondria in TG Hearts

EM profiles of cardiac myocytes from hTMPK TGs and WTs treated with AZT, RCV, or respective vehicle were assessed from formalin-fixed cardiac tissues. Ultrastructural features in mitochondrial structure from vehicle-treated TGs (both CMC and saline) were essentially unchanged compared to WT littermates (Figure 3a and b). Likewise, mitochondria from AZT- or RCV-treated WTs appeared unremarkable when compared to their vehicle-treated littermates. Mitochondria of cardiac myocytes from AZT-treated TGs, however, exhibited disruption of sarcomeres and tubules, mitochondrial swelling, and decreased cristae density compared to all other cohorts (Figure 3a, lower right panel). These findings correlate with decreased mtDNA abundance in cardiac samples from AZT-treated TGs and together suggest disruption of mitochondrial biogenesis. In contrast, mitochondria from RCV-treated TGs exhibited essentially no ultrastructural changes (Figure 3b, lower right panel) and appeared identical to WT littermates.

ECHO Evalution of LV Mass in Hearts of TMPK TGs with and without Antiretroviral Therapy

Both LV mass and LVEDD offer direct assessments of cardiac function and mass. An increase in either parameter is characteristic of cardiac dysfunction and suggestive of cardiac hypertrophy (increased LV mass) and/or dilation (increased LVEDD). ECHO data were recorded at termination of studies for individual mice from each cohort of hTMPK TGs and WTs with and without AZT or RCV treatments (35 days). LV mass for vehicle-treated (CMC or saline) TGs remained unchanged compared to WT littermates (Figure 4a and b, $P > 0.05$). AZT treatment led to increased LV mass in both WTs and TGs compared to vehicle (CMC)-treated WT (Figure 4a, $P < 0.05$), suggesting LV hypertrophy. No significant change in LV mass was found after RCV treatment compared to vehicle (saline)-treated WTs (Figure 4b). LV cavity volume, reflected in LVEDD, remained unchanged for all cohorts, including AZT-treated TGs (data not shown), suggesting no left ventricular dilation.

Histological Analysis

H&E-stained microscopic sections of hearts from TGs and WTs with and without AZT or RCV treatment were analyzed comparatively (original amplification $\times 40$). Representative images from each cohort show no significant myocytolytic changes in any of the heart tissues including treatment with AZT (Figure 5a) or RCV (Figure 5b).

DISCUSSION

TG mice with cardiac-targeted gene expression are useful and powerful tools to define features of cardiac dysfunction,[30] antiretroviral-associated cardiomyopathy,[31] genetic changes related to mitochondrial function,[32] and defects in mitochondrial biogenesis relate to drug toxicity.[33] We report here a novel *in vivo* murine model with cardiac-targeted

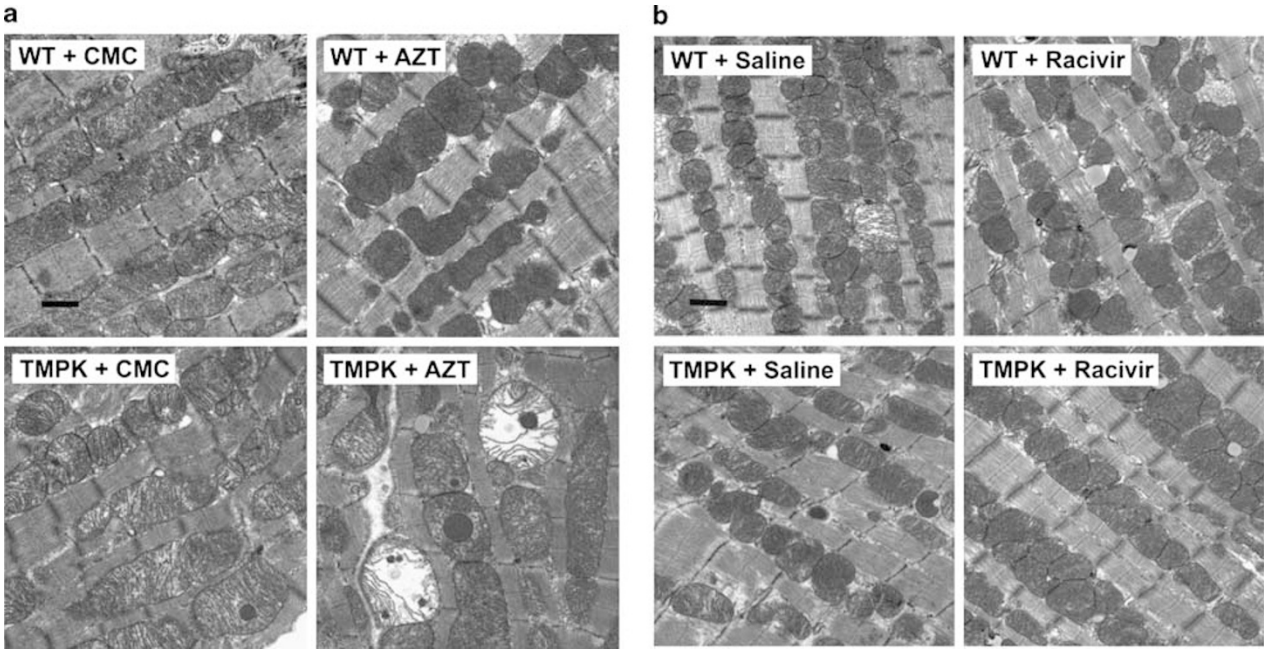

Figure 3 Electron photomicrographs of the mitochondria from cardiac myocytes of TMPK TG and WT treated with AZT or RCV: Representative TEM profiles of cardiac tissues from '2 × 2' studies of TMPK TGs and WTs treated with AZT (**a**), RCV (**b**), and their respective vehicle controls are shown. Cardiac myocytes from TMPK TGs treated with AZT show disrupted sarcomeres and tubules with mitochondrial swelling and reduced cristae density (**a**, lower right panel) compared to vehicle-treated WTs. (**b**) Changes after treatment with RCV in TGs and WTs were unremarkable. (Original magnification: × 22 300, marker indicates 1 μm.)

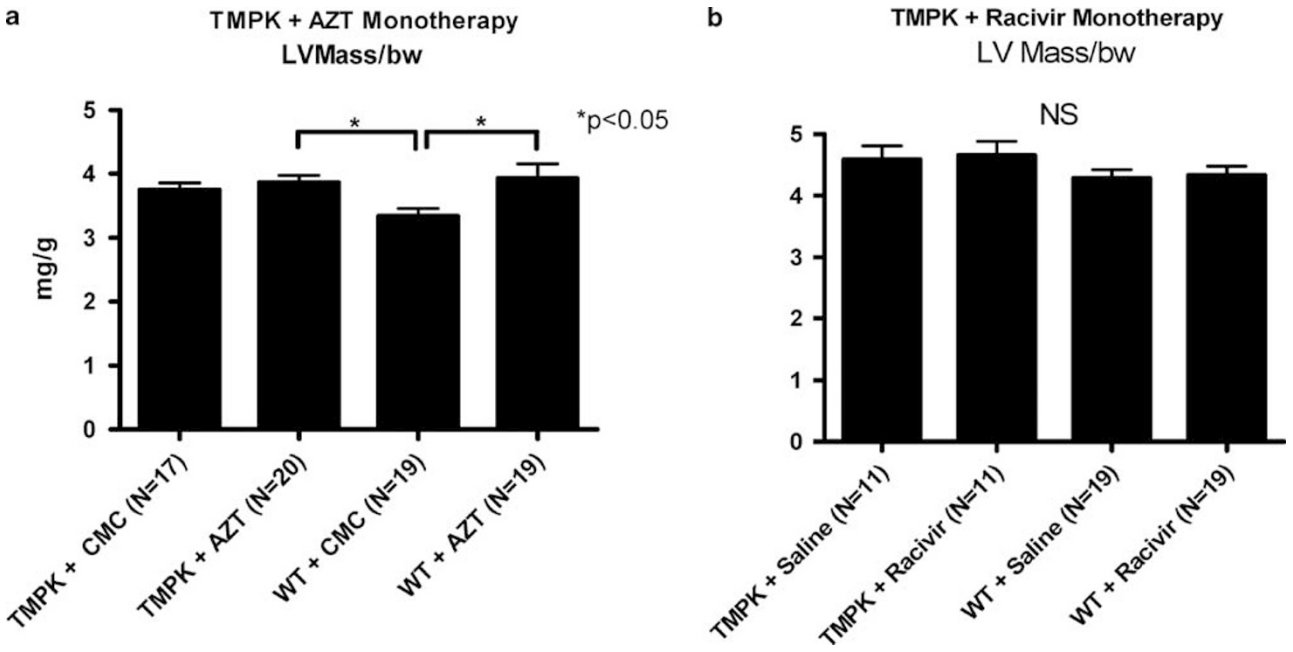

Figure 4 Quantitative analysis of ECHO images. LV mass was determined from ECHO images captured just before termination. Data were normalized to body weight (mg/g) and plotted as mean ± s.e.m. (**a**) AZT treatment increased LV mass in WT and TGs, compared to vehicle-treated WTs ($P<0.05$). (**b**) LV mass remained unchanged in TGs and WTs after RCV treatment (right graph).

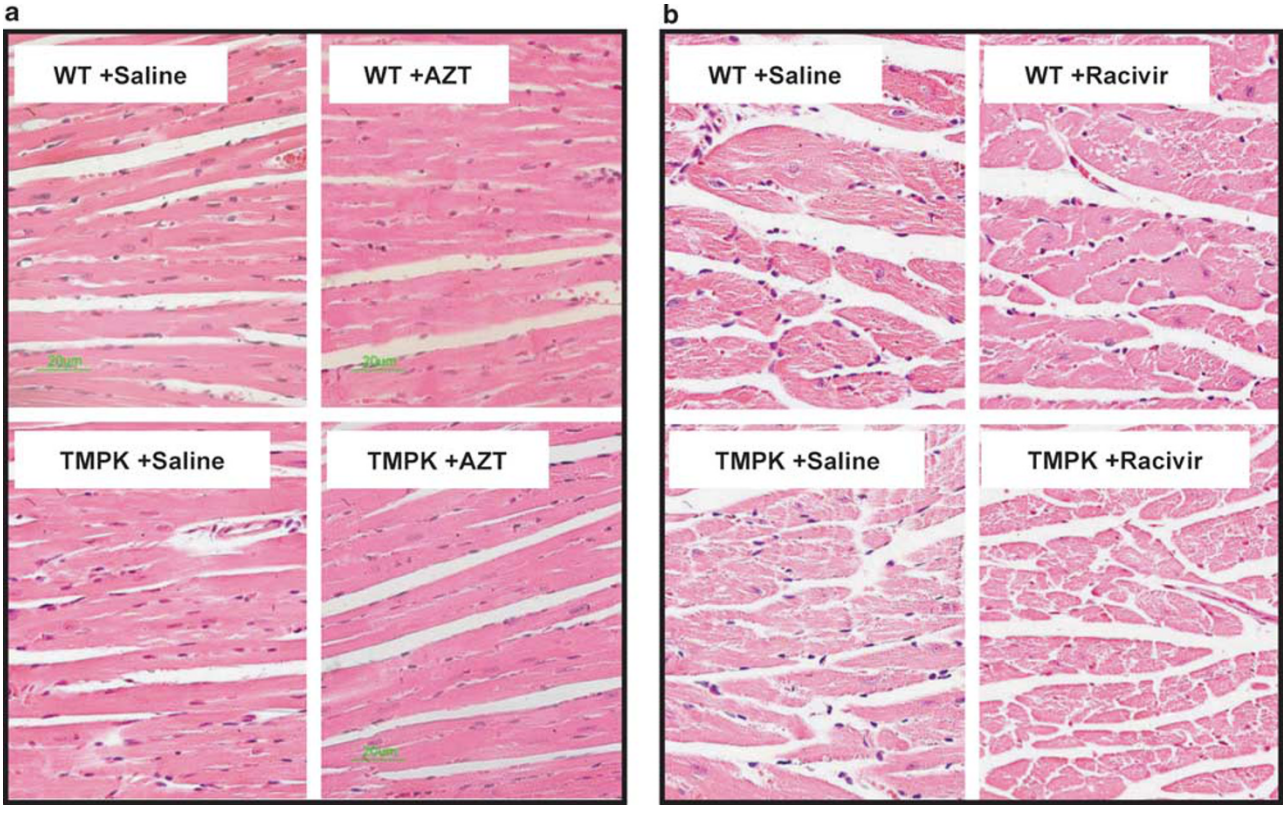

Figure 5 Histological analysis of cardiac tissues from TMPK TGs and WTs treated with AZT or RCV: Parallel H&E-stained slides were made from gender-matched pairs of cardiac tissues after treatment (35 days). All tissues from TMPK TGs and WTs treated with AZT (**a**) or RCV (**b**) showed intact cardiomyocytes with comparable nuclei (original magnification × 40).

overexpression of human TMPK. TMPK is a cell-cycle regulated enzyme, expressed in the S phase.[34] Three individual TG lines were generated. Each encoded multiple copies of the human TMPK gene in addition to the native murine TMPK gene. It was imperative to select a screening method that differentiates hTMPK from the native murine TMPK. The successful approach used specific primers to target tandem regions of α-MyHC promoter and hTMPK (Figure 1a) and a set of probes that would bind to the amplified product. Our method was reproducible, accurate, and resulted in the amplification and detection only of the hTMPK gene (Figure 1b and c). TG line C (possessing the highest gene copy number) was selected because robust overexpression of hTMPK in cardiac muscle was expected to increase phosphorylation of dTMP to dTDP most effectively, and impact mtDNA replication and cardiac function.

TMPK TGs here (vehicle-treated) showed a small yet measurable decrease in mtDNA abundance (Figure 2, $P > 0.05$). Although overexpression of TMPK may cause increased TDP, dNTP pools are tightly regulated and rely on steady state of a reaction. Increasing the abundance of enzyme alone does not necessarily increase flux through a pathway unless substrate is unlimited. Factors such as availability of substrate (dTMP) demand for product (dTTP), and activities of other enzymes in the phosphorylation and dephosphorylation pathways also have a role.

It could be argued that any potential increase in cardiac mitochondrial dTTP abundance caused by TMPK TGs *per se* has only moderate impact, as mentioned earlier, through the effects on other nucleoside kinases and on ribonucleotide reductase. Specifically, *in vitro* studies found dTTP is a feedback inhibitor of human TK 1[35,36] and is a regulator of ribonucleotide reductase.[37] It remains unclear whether these inhibitory mechanisms can occur in quiescent cells, such as in cardiac myocytes.

In contrast, significant mtDNA depletion, mitochondrial ultrastructural damage, and increased LV mass were found in TMPK TGs treated with AZT. Cardiac overexpression of TMPK, the rate-limiting enzyme in the pathway converting AZT to AZTTP, may have resulted in increased AZTTP levels, which can directly inhibit pol γ.[33] However, these experiments offer no direct evidence (ie measurement of dTTP pools) to support this conclusion.

RCV had no impact on TG (or WT) LV mass or mitochondrial ultrastructure. As TMPK kinase is relatively substrate specific for thymidine, the cytidine analog, RCV is not phosphorylated by it. However, RCV treatment of TGs significantly decreased mtDNA abundance (Figure 2b). This latter finding supports (albeit indirectly) the notion of the absence of a universal mechanism of mitochondrial toxicity of NAs.[38] Although AZT led to decreased mtDNA abundance and mitochondrial ultrastructural changes that ultimately impacted cardiac function, the effect of RCV was limited to decreased mtDNA abundance alone.

Overexpression of TMPK may also impact dNTP pool balance. A direct assessment of dNTP pools would help define the role of TMPK on mitochondrial dNTPs. Such measurements were not made in these studies. Future studies using TMPK TGs will directly determine dNTP pools.

Overall, initial studies suggest enhanced TMPK activity by genetically engineered overexpression (TG) renders detectable changes in the mtDNA replication with potentially significant subcellular consequences.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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