HHS Public Access

Author manuscript

Mitochondrion. Author manuscript; available in PMC 2022 November 01.

Published in final edited form as:

Mitochondrion. 2021 November ; 61: 147–158. doi:10.1016/j.mito.2021.09.010.

Remdesivir triphosphate blocks DNA synthesis and increases exonucleolysis by the replicative mitochondrial DNA polymerase, Pol γ

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Abstract

The COVID-19 pandemic prompted the FDA to authorize a new nucleoside analogue, remdesivir, for emergency use in affected individuals. We examined the effects of its active metabolite, remdesivir triphosphate (RTP), on the activity of the replicative mitochondrial DNA polymerase, Pol γ. We found that while RTP is not incorporated by Pol γ into a nascent DNA strand, it remains associated with the enzyme impeding its synthetic activity and stimulating exonucleolysis. In spite of that, we found no evidence for deleterious effects of remdesivir treatment on the integrity of the mitochondrial genome in human cells in culture.

Keywords

remdesivir; COVID-19; mitochondrial DNA; DNA polymerase gamma; antiviral nucleoside analogues

1. INTRODUCTION

Remdesivir (GS-5734) was the first pharmaceutical approved by the U.S. Food and Drug Administration (FDA) for the treatment of COVID-19, under Emergency Use Authorization (1). Remdesivir is a prodrug, which in vivo undergoes conversion to a 1’-cyano (1’-CN) modified adenosine triphosphate analogue (GS-443902) (2,3), here referred to as remdesivir triphosphate (RTP). As a ribonucleotide, RTP acts as a substrate for RNA synthesis and it

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has been demonstrated to be efficiently utilized by viral RNA-dependent RNA polymerases (RdRp), such as those of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or Ebola virus (EBOV) (4,5). Biochemical and structural studies have determined that incorporation of RTP results in delayed RNA-chain termination (4,6) due to a steric clash of the 1'-CN substituent with amino acid side chains within the RNA-binding channel of viral polymerases; in the case of the SARS-CoV-2 RdRp, the clash occurs with Serine 861 (7,8). Notably, reports to date indicate that RTP is selective for viral polymerases and appears to have little to no effect on mammalian DNA and RNA polymerases, which advocates for its application in the treatment of viral infections (5,6,9,10). In fact, the overall safety of remdesivir (i.e., GS-5734) for use in human subjects has been positively evaluated in clinical studies, although some adverse effects have been identified (11–15).

Significant homology with viral counterparts makes the mitochondrial transcription and DNA replication systems prone to cross-reacting with antiviral pharmaceuticals (16–19). Because of this, mitochondrial toxicity is one of the major concerns of applying antiviral nucleoside analogues for human treatment (20). Thus far, little is known about the effects of remdesivir on mitochondrial metabolism and biogenesis. A recent report from the manufacturer of remdesivir (i.e., Veklury®, Gilead Sciences) suggested no considerable risk of eliciting mitochondrial toxicity (21). However, another recent preprint indicated that remdesivir may repress the respiratory activity of cells (22). Of interest, the Division of Applied Regulatory Science of the FDA indicated, in a clinical pharmacology review issued in the process of approval of Veklury® for COVID-19 treatment, that *rhesus* monkeys treated with a high dose of remdesivir (yet lower than that in use for human COVID-19 treatment) exhibited a substantial (26%) loss of mitochondrial (mt)DNA, and suggested a need for further investigation (23).

In this study, we focused on the assessment of the effects of RTP on the activity of human mtDNA polymerase gamma (Pol γ) and the integrity of the human mitochondrial genome. The potential interference with the mtDNA synthesis process is of particular concern, as it may give rise to heritable mtDNA mutations, which may be carried undetected for generations before populating mitochondria to the level that onsets a mitochondrial disease. The gravity of this problem has been recently illustrated in an epidemiological survey, which indicated that even though, with a frequency of ~1 in 5000 individuals, mitochondrial diseases are rare, 1 in ~200 individuals is a carrier of a pathogenic point mutation in mtDNA, making it a prevalent health problem in the human population (24). Therefore, even if pharmaceuticals do not elicit severe mitochondrial toxicity in treated individuals, as seems to be the case for remdesivir, their effects on the maintenance and the integrity of the mitochondrial genome warrant deeper investigation.

Pol γ is the only replicative DNA polymerase in human mitochondria (25). The holoenzyme of Pol γ is a heterotrimer composed of a catalytic subunit (Pol γα) and a homodimeric accessory subunit (Pol γβ). The catalytic subunit belongs to the family A of DNA polymerases and consists of an exonuclease and a polymerase domain. The enzyme possesses DNA polymerase, 3'−5' exonuclease, and 5'-dRP lyase activities (26). In vitro studies demonstrated that Pol γ can incorporate and extend ribonucleotides (rNTPs) during DNA synthesis (27–29). Consistent with this, mtDNA molecules in vivo contain persistent

single ribonucleotides that are not removed due to an apparent lack of dedicated repair pathways (30–34). Notably, rNTPs have been demonstrated to impede DNA synthesis by Pol γ, as upon incorporation they are hardly excised and, at the same time, their extension occurs at a much slower rate, consequently stalling the enzyme (28,35). Taken together, these facts raise the question of whether Pol γ can incorporate RTP into a nascent strand, and whether such putative occurrences may be detrimental to the activity of the enzyme and to the integrity of the mitochondrial genome.

Here we report that, similar to rNTPs, RTP impedes DNA synthesis by Pol γ in vitro when present in excess over deoxyribonucleotides (dNTPs). However, in contrast to rNTPs, RTP is not incorporated into a nascent DNA strand, but remains associated with Pol γ stimulating its exonucleolytic activity. Our structural analysis suggests that upon the positioning of RTP into the polymerase active site, its 1'-CN substituent may interact with residues critical for mismatch sensing and positioning of the nascent strand end (i.e., R853 and Q1102 (36)), which in turn may trigger relocation of the 3'-end of the nascent DNA strand into the exonuclease catalytic site. Notably, the effect of RTP on Pol γ appears to be persistent as, once exposed, Pol γ remains incapable of DNA synthesis despite favorable conditions. However, we found no evidence that remdesivir affects the integrity of the mitochondrial genome in human cells in culture.

2. MATERIALS AND METHODS

2.1. Proteins, nucleic acids and nucleotides

All in vitro experiments in this study were performed with the use of the Pol γ holoenzyme composed of the catalytic subunit Pol γα and the dimeric accessory subunit Pol $\gamma\beta_2$, combined in a 1:1.5 molar ratio. Where indicated, the proof-reading deficient (Exo^-) Pol γα subunit, harboring the D198A and E200A substitutions, was used instead of the wild-type Pol γα. Pol γ subunits were purified in Dr. Laurie Kaguni's Lab (Michigan State University), as described previously (37). Unless otherwise indicated, all in vitro experiments presented in this study were performed with the use of a primer-template DNA substrate composed of a 5'-Cy3-labeled 15 nt-long primer: CGCCAGGGTTTTCCC, and a 44 nt-long template strand: GCACTGGCCGTCGTTTTACGGTCGTGACTGGGAAAACCCTGGCG (see also Figure 1 A). For the staged primer extension assays, presented in Figure 4 B, the described primer-template was used without the fluorescent label, as the primary DNA substrate. The secondary primer-template substrate was composed of the same 5'-Cy3-labeled primer and a 45 nt-long template strand that contained no thymidylate residues downstream of the primer annealing sequence: GAGACCAACACCGACCACGAACCAACAGCAGGGAAAACCCTGGCG.

Deoxyribonucleotides were purchased from Qiagen. ATP was purchased from Promega. dGTP and GTP were purchased from Invitrogen. Remdesivir triphosphate (i.e. GS-443902) was purchased from Biosynth Carbosynth (United Kingdom). Remdesivir nucleoside (GS-441524) was purchased from AdipoGen (USA).

2.2. Primer extension assay

The extension reactions were prepared in a 20 μl reaction mixture containing 50 mM Tris HCl pH 8.5, 20 mM KCl, 10 mM DTT, 4 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 10% glycerol, 5 nM Pol γ holoenzyme, 20 nM DNA substrate, and the indicated amounts of dNTPs, ATP, and RTP. The reactions were carried out at 37 °C for either 10 (Figure 2) or 20 minutes (Figures 1, 4A). The reactions were terminated by the addition of 10x stop solution (100 mM EDTA, 10 % SDS) and 0.5 μg Proteinase K (ThermoFisher Scientific), followed by a 30 minutes incubation at 55 °C and the addition of equal volume of 90% formaldehyde, 50 mM EDTA solution. DNA was denatured at 95°C for 10 minutes and analyzed on 12% (Figures 1, 5) or 20 % (Figure 2) denaturing (7M Urea) polyacrylamide (19:1) gels. The gels were scanned for Cy3 fluorescence in a GE Typhoon Trio+ Variable Mode Imager and the intensity of bands was analyzed with ImageJ (NIH).

2.3. Exonuclease assay

The exonuclease assays (presented in Figure 2 B, also Supplementary Figure 2) were performed under the same conditions as the primer extension assay, in the absence of dNTPs, and in the presence or absence of 0.5 mM RTP or GTP. The reactions were conducted in a master mix and 20 μL aliquots were stopped at the indicated time intervals. DNA products were analyzed on 20% denaturing polyacrylamide gels, as described in the previous section. The density of the bands was quantified by densitometry (ImageJ, NIH). The fraction of the exonucleolysis products was quantified relative to the abundance of all the species detected (i.e., exonucleolysis products + primer). The results were plotted against time and the initial reaction rates were calculated from the slopes of the linear regression fitted to the initial data points, divided by the concentration of the enzyme in the reaction.

2.4. Staged primer extension assay

The staged primer extension assay (presented in Figure 4B) was performed under the same conditions as the standard primer extension assay. However, first, the reaction mixture containing the wild-type Pol γ and 20 nM unlabeled primary DNA substrate, with or without 0.5 mM RTP, ATP, or dATP, was incubated at 37 °C for 10 minutes. Next, 40 nM labeled secondary substrate was added together with 2 mM (total) dNTPs, and the incubation continued for an additional 20 minutes. DNA products were analyzed on 12 % denaturing polyacrylamide gels, as described in the previous section.

2.5. Molecular Modeling

The software Pymol (www.pymol.org) was used to model RTP into the Pol γ catalytic site, to analyze all structural details and to produce the figures. The palm subdomain of Pol γ (residues 868–894 and 1094–1186) and of SARS-CoV-2 RdRp (residues 594–621 and 679–802), in the structures deposited under the PDB codes 4ZTU (36) and 7BV2 (38), respectively, were first aligned under default settings (Supplementary Figures 3A and B). The sugar moieties of remdesivir monophosphate from 7BV2 and of dideoxycytidine triphosphate (ddCTP) from 4ZTU were then aligned separately, allowing the beta and gamma phosphate groups of RTP to be built based on the conformation of ddCTP interacting with the two Mg^{2+} ions at the Pol γ catalytic site (Supplementary Figure 4).

2.6. Fibroblast culture

Primary human (neonatal) dermal fibroblasts (PrimaPure, Genlantis, San Diego, CA) were cultured in Fibroblast Growth Medium (Genlantis, San Diego, CA). Cells were seeded in 6-well plates at a density of 30,000 cells per well and were grown for 3 days. The cells were then grown for an additional 7 days in the presence of 1, 5, 10, 25, and 50 μM remdesivir nucleoside (GS-441524), or in the presence of DMSO in the volume equivalent to that brought in with the addition of 50 μM remdesivir nucleoside (the mock sample). Experiments were carried out in two biological repeats.

2.7. Quantitative-Real Time PCR

Cells were trypsinized, resuspended in 200 μL of PBS buffer containing 0.2 mg/ml Proteinase K, 0.2 % SDS, 5 mM EDTA and incubated for 3 hr at 55°C. Total DNA was isolated by phenol-chloroform extraction and ethanol precipitation. For quantification of mtDNA copy number, 50 ng of DNA isolated from two biological repeats of the remdesivir nucleoside-treated and untreated cells, was amplified by quantitative real-time polymerase chain reaction (Q-RT-PCR; SYBR®Green JumpStart™ Taq ReadyMix™), targeting the 12S mitochondrial gene and the actin nuclear gene with the following primers: 12S FWD 5'-TAGCCCTAAACCTCAACAGT-3', 12S REV 5'-TGCGCTTACTTTGTAGCCTTCAT-3', Actin Q-PCR FWD 5'-TCACCCACACTGTGCCCATCTACGA-3', Actin Q-PCR REV 5'-CAGCGGAACCGCTCATTGCCAATGG-3' (method and primers were adopted from (39)). The C_t values of the mtDNA target were normalized to the C_t values of the nuclear DNA target. Normalized values were then normalized again to the mean normalized values obtained from the RTP untreated cultures. The results were analyzed by one-way ANOVA test followed by post-hoc Dunnett's comparison of the remdesivir nucleoside-treated cells to the untreated cells, using the software GraphPad Prism.

2.8. Detection of the common deletion

The presence of the common deletion (Δ8471–13447 nt) was tested by nested PCR, as described previously (39). Briefly, 100 ng of total DNA isolated from two biological repeats of the remdesivir nucleoside-treated and untreated cells, was amplified by PCR using primers: common deletion PCR1 FWD 5'-AACCACAGTTTCATGCCCATC-3', and common deletion PCR1 REV 5'-TGTTAGTAAGGGTGGGGAAGC-3', for 15 cycles. 1μL of the reaction mixture was next used for the second round of amplification with primers flanking the deletion break points: common deletion PCR2 FWD 5'-ACCCTATAGCACCCCCTCTAC-3', and common deletion PCR2 REV 5'-CTTGTCAGGGAGGTAGCGATG-3', for 30 cycles. As a control of mtDNA presence, a fragment of the 12S mitochondrial gene was amplified (see 2.7 for primer sequences) and visualized by agarose gel electrophoresis. As a positive control, DNA was isolated from fibroblasts cultured as described in section 2.6, in the presence of 50 μM ethidium bromide, which has been previously demonstrated to induce the common deletion in mtDNA (40). The presence of the common deletion in the positive control sample was confirmed by sequencing the product of the nested PCR.

3. RESULTS

3.1. RTP inhibits DNA synthesis by Pol γ.

To examine the effect of RTP on mtDNA synthesis, we first tested its effect on the activity of human Pol γ, in vitro. To that end, we performed a primer extension assay in the presence of dNTPs and increments of RTP (Figure 1). The template DNA strand used in these experiments contained multiple thymidylate residues (Figure 1A), which given that RTP is an analogue of ATP, correspond to putative RTP incorporation sites. We observed no quantitative or qualitative differences between the products generated in the presence or absence of RTP at a concentration equal to that of a single dNTP (hence four-fold lower than the total dNTPs concentration). However, we observed a substantial reduction in the abundance of the DNA products (>80%) when RTP was present in a 2.5-fold excess over total dNTPs concentration (Figure 1B). To assess whether this inhibitory effect is dependent on the ratio of RTP to dNTPs, and not on independent effects of RTP, we performed the experiment in reverse order, using a low uniform RTP concentration and increments of dNTPs. Again, we observed a decrease in the abundance of the DNA products when RTP was present in excess over dNTPs, and no effect of RTP when dNTPs were in excess (Supplementary Figure 1A). These results indicate that RTP competes with dNTPs for the active site of Pol γ and, once bound, impedes its DNA synthesis activity.

Incorporation of RTP by viral RNA polymerases into a nascent RNA strand terminates its elongation, which in primer extension assays appears as an accumulation of shorter rather than full-length products (4,41). Inhibition of Pol γ in our experimental conditions, however, did not result in the accumulation of shorter products (Figure 1B). We considered that this may be due to proof-reading events, which could simply delay the synthesis of full-length products. To evaluate this possibility, we examined the effect of RTP on the exonuclease-deficient variant of Pol γ (Pol γ Exo$^-$). We did not observe any qualitative or quantitative differences between DNA products generated by Pol γ Exo$^-$ in the presence or absence of RTP (Figure 1C, Supplementary Figure 1A). This finding contrasts with the effects observed in the case of viral polymerases and implies that the inhibitory effect of RTP on Pol γ activity depends on its exonucleolytic activity.

3.2. RTP stimulates the exonuclease activity of Pol γ.

The exonuclease-dependent inhibition of DNA synthesis by RTP is strikingly similar to the effect of rNTPs on the activity of Pol γ (35). However, the rNTP-induced inhibition of Pol γ results in the accumulation of shorter products, which, again, we did not observe. To the contrary, in the presence of RTP, the shorter DNA products were depleted (indicated by the arrow in Figure 1B). In our experimental conditions, the short DNA products are generated subsequently to the full-length product (Supplementary Figure 1B), which indicates that they are likely products of exonucleolysis. This is also consistent with the absence of such short products when Pol γ Exo$^-$ was used (Figure 1C). Therefore, there could be two alternative reasons for the depletion of the short products in response to RTP: either inhibition or stimulation of the exonuclease activity.

To examine the putative effect of RTP on the exonucleolytic activity of Pol γ, we compared the quantity and quality of products generated in the presence or absence of dNTPs, or in the presence of RTP and dGTP, individually or in combination (Figure 2A). As expected, in the absence of dNTPs we observed a modest increase in the products of exonucleolysis (Figure 2A, compare lanes 1 and 2). In the presence of RTP, Pol γ exhibited elevated exonuclease activity, as evident from the >2-fold increase in the abundance of the exonucleolysis products (Figure 2A, compare lanes 2 and 3). In contrast, Pol γ did not exhibit any considerable exonucleolytic activity in the presence of dGTP (Figure 2A, lane 4), even though it is a mismatch for the first nucleotide in the template strand (see Figure 1A). On the contrary, Pol γ extended the primer by up to three nucleotides, which is likely imposed by a relatively high concentration of dGTP in the assay, as consistent with previous reports (42,43) (although we also cannot exclude the possibility that this may be due to a contamination of our dGTP stock). Similarly, we observed no changes in primer excision when dGTP was used in equimolar combination with RTP. These results imply that RTP stimulates the exonucleolytic activity of Pol γ, which however, can be mitigated by the presence of dNTPs, in agreement with the previous observation (Figure 1B). To quantify this stimulatory effect, we compared the efficiency of exonucleolysis by Pol γ, in the presence or absence of RTP, in a time course experiment (Supplementary Figure 2A). We estimated that in the presence of RTP, the initial rate of exonucleolysis increased ~2-fold; i.e. from 0.01 s^{-1} in the absence of RTP (which is consistent with the previously reported value (44)) to 0.018 s^{-1} in the presence of RTP (Figure 2B).

In addition, using the same experimental approach, we assessed whether Pol γ is capable of incorporating RTP into a nascent DNA strand. To this end, we examined products generated by Pol γ Exo^- in the presence of RTP, or dATP as the incorporation reference, at a single nucleotide resolution (Figure 2C). We observed no primer extension by Pol γ Exo^- in the presence of RTP, while the primer was efficiently extended in the presence of dATP. This indicates that Pol γ does not incorporate RTP into a nascent DNA strand, which corroborates previous reports (9,21). Considering these results, there is no reason to assume that the inhibitory effect of RTP is contingent on the sequence of the template DNA strand. To evaluate this, we examined the ability of Pol γ to extend a primer over a DNA template that contained no thymidylate residues, in the presence and absence of RTP. Indeed, we observed that RTP inhibited DNA synthesis even in the absence of thymidylate residues in the DNA template (Supplementary Figure 1C). This indicates that the inhibition of Pol γ activity by RTP is not sequence specific.

Our observations indicate that there are significant differences between the effect of RTP and rNTPs on Pol γ activity. In particular, RTP is not incorporated into a nascent DNA strand by Pol γ, and yet it enhances its exonucleolytic activity, whereas rNTPs have been demonstrated to be incorporated into a nascent strand by Pol γ and are hardly excised (27,28). To evaluate these differences in our experimental conditions, we examined DNA products generated by the wild-type or the Exo^- Pol γ in the presence of ATP, or dATP as a reference, at a single nucleotide resolution (Figure 2D). Consistent with the previous reports, we observed that both the wild-type and the Exo^- variants of Pol γ extended the primer in the presence of ATP efficiently (Figure 2D lanes 3, 4). Notably, we observed no impact of ATP on the exonucleolytic activity of Pol γ (Figure 2D lanes 1, 3). Furthermore, we tested

the effect of GTP on the primer exonucleolysis. GTP is a mismatch for the first nucleotide to be incorporated and for the last six nucleotides of the primer (−1 to −6), which prevents 'refilling' of the excision products by Pol γ (Supplementary Figure 2B). As opposed to RTP, the rate of primer excision by Pol γ in the presence of GTP decreased to 0.006 s^{-1} (Figure 2B). Together, these results confirm the significant differences between the effect of RTP and rNTPs on Pol γ activity.

3.3. Structural analysis suggests the 1'-CN group of RTP triggers conformational changes that lead to the increased exonucleolytic activity of Pol γ.

To gain insight into how RTP could interfere with DNA synthesis and stimulate the exonucleolytic activity of Pol γ, we compared the available crystal structures of Pol γ in a ternary complex (36), and the cryo-electron microscopy structure of the SARS-CoV-2 RdRp (the NSP12/8/7 complex) associated with RTP and a primer-template RNA (38). Analyses of the palm subdomain of the catalytic subunits Pol γα and NSP12 demonstrated a striking structural similarity (Supplementary Figure 3A). Amino acid sequence alignment indicated that the similar architecture of the palm is formed partially by the conserved polymerase motifs A and C (Supplementary Figure 3B). We used such similarity to align the structure of both enzymes (Supplementary Figure 3C) and model RTP into the catalytic site of Pol γα. Notably, RTP accommodates into the catalytic site without any apparent impediment, in a conformation that would favor nucleotide incorporation (Supplementary Figure 4). However, we speculate that Pol γα residue E895, and perhaps also Y951 and Y955, known to be essential for recognition of the correct incoming nucleotide (45–47), may be able to sense the 2'-OH of the RTP sugar moiety and prevent phosphodiester bond formation (Figure 3B). However, this sensor mechanism is apparently not fully efficient against rNTP incorporation in vitro, as we here and others have shown (Figure 2D, (27)). Although NSP12 D623 appears to be analogous to Pol γα E895, its positioning in the catalytic site of the viral enzyme suggests it facilitates RTP (and rNTPs) incorporation (38). In addition, NSP12 does not have residues analogous to Pol γα Y951 and Y955 (38).

The main structural difference between RTP and ATP reside in the presence of the cyano substituent bound covalently to the C1' of the RTP sugar moiety (Figure 3A). Our analysis suggests that when RTP is positioned in the pol catalytic site of Pol γ, the 1'-CN is in close proximity to the side chains of R853 and Q1102 (and perhaps N1098) (Figure 3C). These residues are critical for sensing the primer-template DNA in the minor groove, as well as the complementarity of the incoming nucleotide (36). It has been suggested that Pol γ uses an energetic coupling network for communication between the pol and exo domains (48). The distortion of the double helix that occurs upon a mismatch is sensed by R853, N1098 and Q1102, triggering cooperative allosteric changes in the enzyme that lead to the repositing of the 3'-end of the nascent DNA strand in the exo catalytic site, which is about 35 Å distant from the pol catalytic site. Our structural predictions suggest that, although RTP is not incorporated into DNA by Pol γ, its simple presence in the pol catalytic site may simulate this cooperative primer-template mismatch response, due to the impact of the 1'-CN substituent on R853, Q1102 and/or N1098. 3'−5' exonucleolysis is then elicited for the removal of nucleotides from the 3'-end of the nascent DNA strand, explaining the results of our biochemical assays showing increased exonucleolytic activity by Pol γ in

the presence of RTP, and the lack thereof in the presence of ribonucleotides (Figure 2B, Supplementary Figure 2).

3.4. The inhibitory effect of RTP on DNA synthesis by Pol γ is persistent.

To better understand the inhibitory effect of RTP on DNA synthesis by Pol γ, we examined whether the RTP-inhibited Pol γ is able to resume DNA synthesis upon addition of excess dNTPs, to a level that promotes DNA synthesis (see Supplementary Figure 5) and renders the remaining RTP no longer inhibitory (see Figure 1B). To this end, we preincubated Pol γ with the DNA substrate, in the presence or absence of RTP, and DNA synthesis was initiated by the addition of excess dNTPs. We found that the presence of RTP in the preincubation step resulted in an over 80% reduction in the abundance of the full-length DNA product (data not shown). In this experimental approach, however, the primer extension may be obstructed by its excision during the preincubation step, which, as we showed, is stimulated in the presence of RTP. Therefore, we also performed a similar experiment, in which Pol γ was either preincubated with RTP and then added to the reaction mixture containing DNA substrate and excess dNTPs, or preincubated alone and then added to the reaction mixture containing DNA substrate, RTP, and excess dNTPs. In this case as well, we observed that preincubation with RTP resulted in about 80% reduction in the product abundance (Figure 4A). To compare the inhibitory effect of RTP with that of rNTPs, we performed the same experiment using ATP instead of RTP. We observed that preincubation with ATP inhibited DNA synthesis to a similar extent as RTP (Figure 4A). In addition, we tested the effect of preincubation with dATP and observed no significant effect on the DNA synthesis efficiency (Figure 4A). These results indicate that once bound, both RTP and rNTPs remain associated with the enzyme for an extended time, inhibiting DNA synthesis even in the presence of high dNTPs concentration.

To investigate this further, we performed a staged primer extension assay, in which Pol γ was initially incubated with an unlabeled “primary” DNA substrate in the presence or absence of RTP, ATP, or dATP. Next, an excess of dNTPs was added to the reaction together with a labeled “secondary” DNA substrate (Figure 4B). In this approach, the DNA product detection depends on the capacity of Pol γ to ‘recover’ from the RTP or ATP-induced inhibition on one DNA substrate and to reinitiate DNA synthesis on a new DNA substrate, which is favored by the addition of excess dNTPs in the second stage. Preincubation of Pol γ with the primary DNA substrate (in the absence of RTP, ATP, or dATP) had no significant impact on its ability to generate a product over the secondary DNA substrate (compare the control and “-” lanes in Figure 4B). The presence of ATP in the preincubation stage resulted in a modest reduction of the final product abundance, by ~20%. In contrast, the presence of RTP in the preincubation stage resulted in a dramatic reduction of the product abundance, by over 80%. The presence of dATP in the preincubation step had no significant impact on the product abundance. This indicates that, in contrast to ribo- and deoxyribonucleotides, once bound, the inhibitory effect of RTP on the DNA synthesis activity of Pol γ is persistent.

3.5. Remdesivir has no apparent deleterious effect on the maintenance and integrity of the mitochondrial genome.

Our structural analysis suggests that the cyano substituent of remdesivir may impact the R853 and Q1102 residues once bound in the Pol γ active site (Figure 3C). Alanine substitution of Q1102 impedes the polymerase activity of Pol γ in vitro and results in early developmental arrest in flies (49). No cases of Q1102 mutations in humans have been reported to date (50), which suggests that changes to this residue may be lethal. Mutations of the R853 residue result in the accumulation of mitochondrial DNA deletions and have been linked with the development of Parkinson's and Alpers diseases (51,52). Conversely, treatment with antiviral nucleosides, which affect Pol γ activity, often leads to accumulation of mtDNA deletions, commonly the deletion of a 4977 bp segment, spanning from 8471 to 13447 nt (53,54). Prompted by these reports, we tested whether remdesivir may be deleterious to the mitochondrial genome in proliferating human cells in culture. For this purpose, we used primary human (neonatal) dermal fibroblasts (HDFs). Fibroblasts have been demonstrated to be a suitable model for the assessment of mitochondrial performance and the integrity of the genome (55–58). Cells were grown for three days, after which the media was supplemented with remdesivir nucleoside (GS-441524; 0, 1, 5, 10, 25, or 50 μM) and cells were grown for an additional seven days before collection. First, we examined mtDNA copy number in the treated and untreated cells by quantitative real-time PCR and found it to be marginally but statistically significantly elevated in all the remdesivir nucleoside-treated cultures (Figure 5A). We also tested the effect of remdesivir prodrug (GS-5734) on mtDNA copy number in a skeletal muscle fibroblast cell line, rhabdomyosarcoma RC13. The cells were grown for 15 days in the presence of 0, 1, and 2 μM remdesivir, but we observed no significant differences in mtDNA content between them (Supplementary Figure 6). Next, to assess the effect of remdesivir on the integrity of the mitochondrial genome, we tested the presence of the common deletion in the remdesivir nucleoside-treated HDFs, by nested PCR. However, we detected no common deletions in any of the samples analyzed (Figure 5B).

4. DISCUSSION

The COVID-19 pandemic generated an immediate need for effective antivirals, of which remdesivir was the first approved by the FDA for emergency use. Given that mtDNA synthesis deficiency and related mitochondrial toxicity are common side effects of therapies engaging antiviral nucleoside analogues (16,17,19,59), we sought to determine the effect of RTP on the activity of Pol γ and the maintenance of the mitochondrial genome in proliferating cells. Considering that previous reports on the subject were either performed by, or tied to the manufacturer of Veklury®, Gilead Sciences (9,10,21), to our knowledge this report is the first independent assessment of the effects of RTP on Pol γ.

We demonstrated here that RTP has an inhibitory effect on the DNA synthesis activity of Pol γ. This effect, however, occurs when the concentration of RTP exceeds that of dNTPs (Figure 1B). In the treatment of COVID-19, administration of the first 200 mg dose of remdesivir results in plasma $C_{max} \cong 9$ μM. Remdesivir is rapidly ($T_{1/2} \cong 1$h) converted to the more stable remdesivir nucleoside monophosphate (plasma $T_{1/2} \cong 24$h),

which can be phosphorylated inside the cells to the active triphosphate form. The estimated plasma concentration of the monophosphate on the first day of treatment is $C_{max} \cong 0.5$ μM, and is maintained at approximately this level through the five days of treatment (3). This concentration is significantly lower than the intracellular concentration of total dNTPs, which depending on the cell cycle phase and cell type, ranges from ~5 to >50 μM (35,60). Hence, according to our observations, remdesivir in the COVID-19 treatment regimen is unlikely to impede mtDNA synthesis. In agreement with this, we found no deleterious changes to the mitochondrial genome integrity and maintenance in remdesivir-treated cells (Figure 5, Supplementary Figure 6), which is consistent with the recent report by Gilead Sciences (21). Notably, the dependance of the inhibitory effect on the ratio to dNTPs resembles the effect of rNTPs, which also inhibit the synthetic activity of Pol γ only when in excess over dNTPs (35). The effect of rNTPs was proposed to be the cause of mtDNA instability reported in patients that suffer from defects in the mitochondrial dNTP supply, such as mutations in thymidine kinase 2 or deoxyguanosine kinase (28,35,61,62). Therefore, by analogy, patients with defects in mitochondrial dNTP supply may be more susceptible to deleterious effects of RTP, which warrants further studies.

Somewhat concerning are our results indicating that the inhibition of the DNA synthesis activity of Pol γ by RTP is rarely reverted by excess dNTPs (Figure 4A). This essentially implies that at a low RTP to dNTPs ratio, Pol γ inhibition events are still likely to occur but are just too infrequent to be detected in our experimental conditions. The intracellular half-life of RTP is relatively long ($T_{1/2} \cong 40$h (3)), thus the probability of its interference in mtDNA synthesis is considerable. Furthermore, the results of the staged primer extension assays (Figure 4B) suggest that, once bound, the inhibitory effect of RTP on the synthetic activity of Pol γ is particularly persistent. RTP inhibits the synthetic activity of Pol γ on the strand at which the inhibition first occurred to an extent comparable with ATP (Figure 4A). However, while after the inhibition by ATP, Pol γ can restart synthesis on a new DNA substrate relatively efficiently (with a reduction of only ~20% compared to control, Figure 4B), the inhibition by RTP renders Pol γ virtually incapable of restarting DNA synthesis on a new DNA substrate (reduction of >80% compared to control, Figure 4B). In our opinion, this difference results from the inability of Pol γ to incorporate RTP (Figure 2C), which otherwise would enable the enzyme to leave the ‘problematic molecule behind’ and reinitiate the synthesis on a new DNA substrate, as appears to be the case here for ATP. Instead, RTP remains associated with the enzyme blocking its activity in the next rounds of the DNA substrate turn over. Alternatively, the persistent inhibitory effect of RTP could result from the inability of the affected Pol γ to dissociate from the primary DNA substrate. However, we find this unlikely because we observed no change in the quantity of DNA substrate hydrolyzed by Pol γ in our exonuclease assays (Figure 2B), which implies that substrate turnover is not affected (i.e., not reduced) in the presence of RTP.

Despite the concerning in vitro observations, we found no direct evidence for defects in the integrity of mtDNA in remdesivir nucleoside-treated fibroblasts (Figure 5B). It is possible that sporadic inhibition of mtDNA synthesis by RTP is mitigated in vivo by mitochondrial DNA replication rescue mechanisms. In fact, it has recently been demonstrated that stalling of Pol γ upon incorporation of chain-terminators can be rescued by a restart mechanism that entails advantageous repriming by PrimPol (63). Notably, the loss of PrimPol function

has been recently associated with the development of mitochondrial toxicity in response to tenofovir in an HIV+ patient and in cultured cells (64). Similar to remdesivir, tenofovir is an antiviral nucleoside, which inhibits Pol γ relatively weakly. Its exacerbated toxicity in the absence of PrimPol has been attributed to the loss of capacity for the fork rescue during mtDNA replication (64). It is possible that the same rescue mechanism alleviates the inhibition of mtDNA synthesis in response to RTP. Further studies on the effects of RTP in the context of PrimPol deficiency would help to address this possibility.

The only effect of RTP on mitochondrial DNA maintenance that we observed was a modest increase in mtDNA copy number in proliferating fibroblasts (Figure 5A). An increase in mtDNA copy number was also earlier observed in HepG2 cells treated with 10 μM remdesivir for 10 days (21). Upregulation of mtDNA replication is often triggered by mitochondrial deficiencies (65,66), therefore, it is possible that this is elicited in remdesivir nucleoside-treated cells to compensate for incidental delays in mtDNA replication. On the other hand, it seems from previous studies that the extent of the toxicity of antiviral nucleosides is related to their capacity to inhibit exonucleolysis by Pol γ (19,67). Hence, the increase in Pol γ exonucleolysis upon RTP binding may simply not be deleterious to the mitochondrial genome in vivo. In fact, an increase in the exonuclease activity over polymerase activity in DNA polymerases, including Pol γ, often has an anti-mutator effect (68,69). Of interest, it has been demonstrated that compound heterozygote flies that express polymerase-deficient Pol γ from one allele and exonuclease-deficient Pol γ from the other allele, are viable and apparently normal, while homozygotes expressing the deficient variants of Pol γ arrest at the larval stage of development (49). This indicates that the Pol γ variants, although incapable of maintaining mtDNA separately, together can complement individual deficiencies and promote sufficient mtDNA synthesis to sustain development. In the context of our study, this suggests that the increase in exonucleolytic activity, at the expense of polymerase activity, in the fraction of Pol γ molecules bound by RTP may be compensated by the remaining unaffected molecules, and overall have no effect on mtDNA maintenance in vivo.

Our structural analysis suggests that the cyano substituent of RTP positions against the side chains of the R853 and Q1102 residues of Pol γ (Figure 3C). The 1’-CN substituent is relatively large and forms a negative dipole at its nitrogen terminus, which is likely to affect the spatial conformation of amino acid side chains in its close proximity. The R853 and Q1102 residues play a critical role in the positioning of the primer-template in the active site of Pol γ and recognition of the incoming nucleotide (36,52). Thus, their steric distortion may have profound consequences on the activity of Pol γ. R853 facilitates coordination of the incoming nucleotide and the −1 base pair site of the primer template (36), which precedes phosphodiester bond formation. Substitution of R853 with glutamine results in a radical loss of polymerase activity (70). Given the similar, polar character of the cyano group, the effect of its positioning against R853 may resemble that of the glutamine substitution, which in turn could explain the loss of the polymerase activity of Pol γ upon RTP binding. Interference with the R853 side chain could also explain why in contrast to rNTPs, RTP is not incorporated into the nascent DNA strand. However, given that R853 facilitates steps prior to the phosphodiester bond formation, the distortion of R853 side chain is unlikely to explain the increase in the exonucleolytic activity. Somewhat in agreement

with this, it has been demonstrated that alanine substitution of the homologous R668 residue in DNA polymerase I of *E. coli* (pol I) does not alter the exonucleolytic activity of the enzyme, despite the loss of polymerase activity (71).

The effect of RTP on the exonucleolytic activity of Pol γ can, however, be explained by an interaction of its cyano group with the Q1102 residue. In vitro, its alanine substitution results in an increase in exonuclease activity with concomitant reduction of the polymerase activity of Pol γ (49), which is strikingly similar to the effect of RTP. Of interest, the Q1102 residue corresponds to the Q849 of pol I, which alanine substitution imparts an anti-mutator effect on DNA replication (72). Induction of the anti-mutator effect by the positioning of the cyano group against the Q1102 side chain could explain the lack of deleterious effects of remdesivir in vivo reported in this and other studies (21). On the other hand, homozygous flies that express Pol γ variants corresponding to the Q1102A substitution in the human enzyme fail to synthesize mtDNA efficiently and arrest at the larval stage of development, while heterozygotes develop normally (49). This indicates that disturbing the Q1102 residue is in fact deleterious to mtDNA maintenance, which, however, can be compensated by concomitant expression of wild-type molecules. By analogy, RTP is not deleterious in vivo likely because its effect is limited to a fraction of Pol γ molecules (due to high intracellular level of dNTPs) and can be compensated by unaffected molecules. Notably, in vitro, the polymerase deficiency of the Q1102A variant of Pol γ is relatively modest and detected only during processive DNA synthesis (the mini-circle assay) (49). This contrasts with our observation that pre-bound RTP impedes the synthesis over a short 29 nt template (Figure 1B). Therefore, overall, our results seem to be more consistent with a synergistic effect of the cyano group on both the R853 and Q1102 residues, rather than on any of them individually.

The inhibitory effect of RTP on DNA synthesis by Pol γ is exonuclease activity-dependent, as the Exo$^-$ variant of Pol γ is unaffected by RTP (Figure 1C), which is similar to the effect of rNTPs (35). Although certain predictions have been made (52), the mechanism of the primer partitioning between the pol and exo active sites of Pol γ remains unknown. More recently, Sowers et al. (48) have proposed that primer partitioning by Pol γ may occur via long-range intramolecular connectivity that takes place through a diffuse cooperative network involving non-adjacent amino acid residues. This may be invoked to explain the mechanistic basis of the dependency we report here, as R853 and Q1102 appear to be important parts of a region of Pol γ that is locally perturbed upon binding by different antiviral inhibitors. This perturbation supposedly spreads, strongly affecting the residues around the exo active site of the enzyme (48). Such hypothesis can also explain how alanine substitutions of the conserved residues S719, Y720 and W721 in the thumb subdomain of *Drosophila* Pol γ (S799, F800 and W801 in the human enzyme) completely abolish pol activity and at the same time increase exonucleolysis three-fold (73). In addition, it is known that the transfer of the primer end between the two active sites occurs during a single binding event (44). This intramolecular transfer occurs continuously during processive DNA synthesis, which actually limits the motor function of Pol γ (74). Studies on other DNA polymerases indicated that transfer of the primer end between the two active sites depends on the presence of magnesium ions in each of the sites. In the absence of magnesium ions in the exo site, the primer remains in the pol site (75). The mutations in the Pol γ Exo$^-$

(D198A and E200A) variant used in this study disable binding of the magnesium ions in the exo site, which suggests that the transfer of the primer end to the exo site in this variant may not occur and the primer remains in the pol site as the enzyme operates. In agreement with this, Pol γ Exo$^-$ exhibits greater motor power than the wild-type enzyme, which is reflected in its capacity for a relatively robust strand-displacement synthesis (which is otherwise negligible) (74,76). The lack of the intramolecular primer shuttling in Pol γ Exo$^-$ explains well the lack of inhibitory effect of rNTPs on its DNA synthesis activity; i.e., Pol γ Exo$^-$ does not back track upon incorporation of rNTP and thus does not stall (35). However, because Pol γ does not incorporate RTP (Figure 2 C), the explanation of its effect is not straight forward. Perhaps in the case of Exo$^-$, the lack of the primer end partitioning and the resulting increase of the motor power enforces the dissociation of RTP from the pol active site enabling continuation of synthesis. Clearly, more studies on the subject are needed to understand the phenomenon of the exonuclease-dependent inhibition of Pol γ by RTP and rNTPs.

Our findings may seem to contradict previous reports, which suggested no adverse effects of RTP on the activity of Pol γ (9,10,21). However, our in vitro experiments were conducted under conditions different than previously, including the concentration of dNTPs, RTP and Pol γ used. Hence, our results are hardly comparable and not necessarily contradictive, especially given that we similarly did not observe any deleterious effects in vivo (21). On the other hand, our study identifies that the ratio to dNTPs, rather than the independent concentration of RTP, is the factor determining Pol γ inhibition, and this stands in contrast to the previous studies, which identified the IC_{50} of RTP for Pol γ to be >200 μM in an assay including ~6μM total dNTPs (9,10). The IC_{50} values in those studies were estimated by a nucleotide incorporation assay, which, to our understanding, was carried out for 60 minutes to assure the consumption of <10% of the radiolabeled substrate (9,10). Per our estimation, this corresponds to the incorporation of <1000 nts per one Pol γ holoenzyme throughout 60 minutes, thus <0.3 nt*s^{-1}. This is from 20 to 150-fold lower than the incorporation rates reported for the wild-type Pol γ (77,78). Therefore, we find it very likely that dNTPs "run out" during this 1h incubation time, allowing the exonucleolytic activity of Pol γ to efficiently take place, skewing the results. We also noted that the assays were performed such that Pol γ and RTP were preincubated prior to the initiation of the reaction by the addition of dNTPs and Mg^{2+} (10). This resembles our experiments in which we showed that preincubation of Pol γ with RTP inhibits mtDNA synthesis despite the later addition of excess dNTPs (Figure 4A). Therefore, it is possible that in the reported experiments the synthetic activity of Pol γ was generally low due to pre-binding of RTP. Alternatively, the differences may result from the Pol γ preparation.

5. CONCLUSION

In conclusion, our study is largely in agreement with previous reports indicating that the potential for deleterious effects of remdesivir on the maintenance of mtDNA, resulting in mitochondrial toxicity, is generally low. However, our in vitro data does raise concerns about the function of Pol γ under conditions prone to dNTP levels fluctuation, during SARS-CoV-2 infection, which to our knowledge has yet to be studied in detail. The fact that mitochondrial function is affected in SARS-CoV-2-infected human monocytes, with

elevated levels of mitochondrial reactive oxygen species being produced (79), provides strong evidence that Pol γ and mtDNA maintenance may be under conditions much more stringent than those that we and others have tested. Further studies are certainly needed to assure that the safety of remdesivir treatment against COVID-19 applies to the various cell types in the human body and to the abundant genetic backgrounds of the human population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Dr. Laurie Kaguni (Michigan State University) for the gift of Pol γ subunits and a critical review of the manuscript. We thank Dr. Giuseppe Cannino (University of Padua) for help with setting up cell culture experiments. This study was supported by the National Institute Of General Medical Sciences of the National Institutes of Health under Award Number R15GM139104. We thank the University of Alabama at Birmingham, Vision Science Research Center (UAB VSRC), Molecular & Cellular Analysis Core for allowing us to use their GE Typhoon Trio+ Variable Mode Imager for quantification of fluorescent signals on polyacrylamide gels. The UAB VSRC cores are supported by NIH grant P30 EY003039. GLC was partially supported by a grant from the Auburn University at Montgomery Research Grant-in-Aid Program. MTO acknowledges funding from the Sao Paulo Research Foundation (grant number 2017/04372-0). MJY acknowledges support from the National Institute of Environmental Health Sciences (5R00ES022638-04) and start-up funds from SIU School of Medicine. MMR is supported by an SIU Carbondale Doctoral Research Fellowship. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

REFERENCES

1. US Food and Drug Administration (2020): Emergency Use Authorization (EUA) for emergency use of remdesivir for the treatment of hospitalized 2019 coronavirus disease (COVID-19) patients. https://www.fda.gov/media/137564/download.
2. Eastman RT, Roth JS, Brimacombe KR, Simeonov A, Shen M, Patnaik S, and Hall MD (2020) Remdesivir: A Review of Its Discovery and Development Leading to Emergency Use Authorization for Treatment of COVID-19. ACS Cent Sci 6, 672–683 [PubMed: 32483554]
3. Jorgensen SCJ, Kebriaei R, and Dresser LD (2020) Remdesivir: Review of Pharmacology, Pre-clinical Data, and Emerging Clinical Experience for COVID-19. Pharmacotherapy 40, 659–671 [PubMed: 32446287]
4. Gordon CJ, Tchesnokov EP, Woolner E, Perry JK, Feng JY, Porter DP, and Götte M (2020) Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency. J Biol Chem 295, 6785–6797 [PubMed: 32284326]
5. Tchesnokov EP, Feng JY, Porter DP, and Götte M (2019) Mechanism of Inhibition of Ebola Virus RNA-Dependent RNA Polymerase by Remdesivir. Viruses 11
6. Warren TK, Jordan R, Lo MK, Ray AS, Mackman RL, Soloveva V, Siegel D, Perron M, Bannister R, Hui HC, Larson N, Strickley R, Wells J, Stuthman KS, Van Tongeren SA, Garza NL, Donnelly G, Shurtleff AC, Retterer CJ, Gharaibeh D, Zamani R, Kenny T, Eaton BP, Grimes E, Welch LS, Gomba L, Wilhelmsen CL, Nichols DK, Nuss JE, Nagle ER, Kugelman JR, Palacios G, Doerffler E, Neville S, Carra E, Clarke MO, Zhang L, Lew W, Ross B, Wang Q, Chun K, Wolfe L, Babusis D, Park Y, Stray KM, Trancheva I, Feng JY, Barauskas O, Xu Y, Wong P, Braun MR, Flint M, McMullan LK, Chen SS, Fearns R, Swaminathan S, Mayers DL, Spiropoulou CF, Lee WA, Nichol ST, Cihlar T, and Bavari S (2016) Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. Nature 531, 381–385 [PubMed: 26934220]
7. Kokic G, Hillen HS, Tegunov D, Dienemann C, Seitz F, Schmitzova J, Farnung L, Siewert A, Höbartner C, and Cramer P (2021) Mechanism of SARS-CoV-2 polymerase stalling by remdesivir. Nat Commun 12, 279 [PubMed: 33436624]

8. Wang Q, Wu J, Wang H, Gao Y, Liu Q, Mu A, Ji W, Yan L, Zhu Y, Zhu C, Fang X, Yang X, Huang Y, Gao H, Liu F, Ge J, Sun Q, Xu W, Liu Z, Yang H, Lou Z, Jiang B, Guddat LW, Gong P, and Rao Z (2020) Structural Basis for RNA Replication by the SARS-CoV-2 Polymerase. Cell 182, 417–428.e413 [PubMed: 32526208]

9. Siegel D, Hui HC, Doerffler E, Clarke MO, Chun K, Zhang L, Neville S, Carra E, Lew W, Ross B, Wang Q, Wolfe L, Jordan R, Soloveva V, Knox J, Perry J, Perron M, Stray KM, Barauskas O, Feng JY, Xu Y, Lee G, Rheingold AL, Ray AS, Bannister R, Strickley R, Swaminathan S, Lee WA, Bavari S, Cihlar T, Lo MK, Warren TK, and Mackman RL (2017) Discovery and Synthesis of a Phosphoramidate Prodrug of a Pyrrolo[2,1-f][triazin-4-amino] Adenine C-Nucleoside (GS-5734) for the Treatment of Ebola and Emerging Viruses. J Med Chem 60, 1648–1661 [PubMed: 28124907]

10. Feng JY, Cheng G, Perry J, Barauskas O, Xu Y, Fenaux M, Eng S, Tirunagari N, Peng B, Yu M, Tian Y, Lee YJ, Stepan G, Lagpacan LL, Jin D, Hung M, Ku KS, Han B, Kitrinos K, Perron M, Birkus G, Wong KA, Zhong W, Kim CU, Carey A, Cho A, and Ray AS (2014) Inhibition of hepatitis C virus replication by GS-6620, a potent C-nucleoside monophosphate prodrug. Antimicrob Agents Chemother 58, 1930–1942 [PubMed: 24419349]

11. Humeniuk R, Mathias A, Cao H, Osinusi A, Shen G, Chng E, Ling J, Vu A, and German P (2020) Safety, Tolerability, and Pharmacokinetics of Remdesivir, An Antiviral for Treatment of COVID-19, in Healthy Subjects. Clin Transl Sci 13, 896–906 [PubMed: 32589775]

12. Mulangu S, Dodd LE, Davey RT, Tshiani Mbaya O, Proschan M, Mukadi D, Lusakibanza Manzo M, Nzolo D, Tshomba Oloma A, Ibanda A, Ali R, Coulibaly S, Levine AC, Grais R, Diaz J, Lane HC, Muyembe-Tamfum JJ, Sivahera B, Camara M, Kojan R, Walker R, Dighero-Kemp B, Cao H, Mukumbayi P, Mbala-Kingebeni P, Ahuka S, Albert S, Bonnett T, Crozier I, Duvenhage M, Proffitt C, Teitelbaum M, Moench T, Aboulhab J, Barrett K, Cahill K, Cone K, Eckes R, Hensley L, Herpin B, Higgs E, Ledgerwood J, Pierson J, Smolskis M, Sow Y, Tierney J, Sivapalasingam S, Holman W, Gettinger N, Vallée D, Nordwall J, Group, P. W., and Team, P. C. S. (2019) A Randomized, Controlled Trial of Ebola Virus Disease Therapeutics. N Engl J Med 381, 2293–2303 [PubMed: 31774950]

13. Goldman JD, Lye DCB, Hui DS, Marks KM, Bruno R, Montejano R, Spinner CD, Galli M, Ahn MY, Nahass RG, Chen YS, SenGupta D, Hyland RH, Osinusi AO, Cao H, Blair C, Wei X, Gaggar A, Brainard DM, Towner WJ, Muñoz J, Mullane KM, Marty FM, Tashima KT, Diaz G, Subramanian A, and Investigators, G.-U.−.−. (2020) Remdesivir for 5 or 10 Days in Patients with Severe Covid-19. N Engl J Med 383, 1827–1837 [PubMed: 32459919]

14. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, Hohmann E, Chu HY, Luetkemeyer A, Kline S, Lopez de Castilla D, Finberg RW, Dierberg K, Tapson V, Hsieh L, Patterson TF, Paredes R, Sweeney DA, Short WR, Touloumi G, Lye DC, Ohmagari N, Oh MD, Ruiz-Palacios GM, Benfield T, Fätkenheuer G, Kortepeter MG, Atmar RL, Creech CB, Lundgren J, Babiker AG, Pett S, Neaton JD, Burgess TH, Bonnett T, Green M, Makowski M, Osinusi A, Nayak S, Lane HC, and Members, A.-S. G. (2020) Remdesivir for the Treatment of Covid-19 - Final Report. N Engl J Med 383, 1813–1826 [PubMed: 32445440]

15. Spinner CD, Gottlieb RL, Criner GJ, Arribas López JR, Cattelan AM, Soriano Viladomiu A, Ogbuagu O, Malhotra P, Mullane KM, Castagna A, Chai LYA, Roestenberg M, Tsang OTY, Bernasconi E, Le Turnier P, Chang SC, SenGupta D, Hyland RH, Osinusi AO, Cao H, Blair C, Wang H, Gaggar A, Brainard DM, McPhail MJ, Bhagani S, Ahn MY, Sanyal AJ, Huhn G, Marty FM, and Investigators, G.-U.−.−. (2020) Effect of Remdesivir vs Standard Care on Clinical Status at 11 Days in Patients With Moderate COVID-19: A Randomized Clinical Trial. JAMA 324, 1048–1057 [PubMed: 32821939]

16. Young CKJ, Wheeler JH, Rahman MM, and Young MJ (2020) The antiretroviral 2',3'-dideoxycytidine causes mitochondrial dysfunction in proliferating and differentiated HepaRG human cell cultures. J Biol Chem

17. Young MJ (2017) Off-Target Effects of Drugs that Disrupt Human Mitochondrial DNA Maintenance. Front Mol Biosci 4, 74 [PubMed: 29214156]

18. Feng JY, Tay CH, and Ray AS (2017) Role of Mitochondrial Toxicity in BMS-986094-Induced Toxicity. Toxicol Sci 155, 2 [PubMed: 27803383]

19. Lim SE, and Copeland WC (2001) Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase gamma. J Biol Chem 276, 23616–23623 [PubMed: 11319228]
20. Lewis W, Day BJ, and Copeland WC (2003) Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. Nat Rev Drug Discov 2, 812–822 [PubMed: 14526384]
21. Xu Y, Barauskas O, Kim C, Babusis D, Murakami E, Kornyeyev D, Lee G, Stepan G, Perron M, Bannister R, Schultz BE, Sakowicz R, Porter D, Cihlar T, and Feng JY (2021) Off-Target. Antimicrob Agents Chemother 65
22. Akinci E, Cha M, Lin L, Yeo G, Hamilton MC, Donahue CJ, Bermudez-Cabrera HC, Zanetti LC, Chen M, Barkal SA, Khowpinitchai B, Chu N, Velimirovic M, Jodhani R, Fife JD, Sovrovic M, Cole PA, Davey RA, Cassa CA, and Sherwood RI (2020) Elucidation of remdesivir cytotoxicity pathways through genome-wide CRISPR-Cas9 screening and transcriptomics. bioRxiv
23. US Food and Drug Administration, Division of Applied Regulatory Science/ Office of Clinical Pharmacology (DARS/OCP); (2020): Application number 214787Orig1s000, Clinical Pharmacology Review(s). https://www.accessdata.fda.gov/drugsatfda_docs/nda/2020/214787Orig1s000ClinpharmR.pdf.
24. Elliott HR, Samuels DC, Eden JA, Relton CL, and Chinnery PF (2008) Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet 83, 254–260 [PubMed: 18674747]
25. Kaguni LS (2004) DNA polymerase gamma, the mitochondrial replicase. Annu Rev Biochem 73, 293–320 [PubMed: 15189144]
26. Ciesielski GL, Oliveira MT, and Kaguni LS (2016) Animal Mitochondrial DNA Replication. Enzymes 39, 255–292 [PubMed: 27241933]
27. Kasiviswanathan R, and Copeland WC (2011) Ribonucleotide discrimination and reverse transcription by the human mitochondrial DNA polymerase. J Biol Chem 286, 31490–31500 [PubMed: 21778232]
28. Berglund AK, Navarrete C, Engqvist MK, Hoberg E, Szilagyi Z, Taylor RW, Gustafsson CM, Falkenberg M, and Clausen AR (2017) Nucleotide pools dictate the identity and frequency of ribonucleotide incorporation in mitochondrial DNA. PLoS Genet 13, e1006628 [PubMed: 28207748]
29. Murakami E, Feng JY, Lee H, Hanes J, Johnson KA, and Anderson KS (2003) Characterization of novel reverse transcriptase and other RNA-associated catalytic activities by human DNA polymerase gamma: importance in mitochondrial DNA replication. J Biol Chem 278, 36403–36409 [PubMed: 12857740]
30. Wanrooij PH, and Chabes A (2019) Ribonucleotides in mitochondrial DNA. FEBS Lett 593, 1554–1565 [PubMed: 31093968]
31. Wanrooij PH, Engqvist MKM, Forslund JME, Navarrete C, Nilsson AK, Sedman J, Wanrooij S, Clausen AR, and Chabes A (2017) Ribonucleotides incorporated by the yeast mitochondrial DNA polymerase are not repaired. Proc Natl Acad Sci U S A 114, 12466–12471 [PubMed: 29109257]
32. Wanrooij PH, Tran P, Thompson LJ, Carvalho G, Sharma S, Kreisel K, Navarrete C, Feldberg AL, Watt DL, Nilsson AK, Engqvist MKM, Clausen AR, and Chabes A (2020) Elimination of rNMPs from mitochondrial DNA has no effect on its stability. Proc Natl Acad Sci U S A 117, 14306–14313 [PubMed: 32513727]
33. Wong-Staal F, Mendelsohn J, and Goulian M (1973) Ribonucleotides in closed circular mitochondrial DNA from HeLa cells. Biochem Biophys Res Commun 53, 140–148 [PubMed: 4582369]
34. Grossman LI, Watson R, and Vinograd J (1973) The presence of ribonucleotides in mature closed-circular mitochondrial DNA. Proc Natl Acad Sci U S A 70, 3339–3343 [PubMed: 4202844]
35. Forslund JME, Pfeiffer A, Stojkovič G, Wanrooij PH, and Wanrooij S (2018) The presence of rNTPs decreases the speed of mitochondrial DNA replication. PLoS Genet 14, e1007315 [PubMed: 29601571]
36. Szymanski MR, Kuznetsov VB, Shumate C, Meng Q, Lee YS, Patel G, Patel S, and Yin YW (2015) Structural basis for processivity and antiviral drug toxicity in human mitochondrial DNA replicase. EMBO J 34, 1959–1970 [PubMed: 26056153]

37. Oliveira MT, and Kaguni LS (2009) Comparative purification strategies for Drosophila and human mitochondrial DNA replication proteins: DNA polymerase gamma and mitochondrial single-stranded DNA-binding protein. Methods Mol Biol 554, 37–58 [PubMed: 19513666]

38. Yin W, Mao C, Luan X, Shen DD, Shen Q, Su H, Wang X, Zhou F, Zhao W, Gao M, Chang S, Xie YC, Tian G, Jiang HW, Tao SC, Shen J, Jiang Y, Jiang H, Xu Y, Zhang S, Zhang Y, and Xu HE (2020) Structural basis for inhibition of the RNA-dependent RNA polymerase from SARS-CoV-2 by remdesivir. Science 368, 1499–1504 [PubMed: 32358203]

39. Phillips AF, Millet AR, Tigano M, Dubois SM, Crimmins H, Babin L, Charpentier M, Piganeau M, Brunet E, and Sfeir A (2017) Single-Molecule Analysis of mtDNA Replication Uncovers the Basis of the Common Deletion. Mol Cell 65, 527–538.e526 [PubMed: 28111015]

40. von Wurmb-Schwark N, Cavelier L, and Cortopassi GA (2006) A low dose of ethidium bromide leads to an increase of total mitochondrial DNA while higher concentrations induce the mtDNA 4997 deletion in a human neuronal cell line. Mutat Res 596, 57–63 [PubMed: 16488450]

41. Jordan PC, Liu C, Raynaud P, Lo MK, Spiropoulou CF, Symons JA, Beigelman L, and Deval J (2018) Initiation, extension, and termination of RNA synthesis by a paramyxovirus polymerase. PLoS Pathog 14, e1006889 [PubMed: 29425244]

42. Longley MJ, Nguyen D, Kunkel TA, and Copeland WC (2001) The fidelity of human DNA polymerase gamma with and without exonucleolytic proofreading and the p55 accessory subunit. J Biol Chem 276, 38555–38562 [PubMed: 11504725]

43. Johnson AA, and Johnson KA (2001) Fidelity of nucleotide incorporation by human mitochondrial DNA polymerase. J Biol Chem 276, 38090–38096 [PubMed: 11477093]

44. Johnson AA, and Johnson KA (2001) Exonuclease proofreading by human mitochondrial DNA polymerase. J Biol Chem 276, 38097–38107 [PubMed: 11477094]

45. Farnum GA, Nurminen A, and Kaguni LS (2014) Mapping 136 pathogenic mutations into functional modules in human DNA polymerase γ establishes predictive genotype-phenotype correlations for the complete spectrum of POLG syndromes. Biochim Biophys Acta 1837, 1113–1121 [PubMed: 24508722]

46. Atanassova N, Fusté JM, Wanrooij S, Macao B, Goffart S, Bäckström S, Farge G, Khvorostov I, Larsson NG, Spelbrink JN, and Falkenberg M (2011) Sequence-specific stalling of DNA polymerase γ and the effects of mutations causing progressive ophthalmoplegia. Hum Mol Genet 20, 1212–1223 [PubMed: 21228000]

47. Graziewicz MA, Longley MJ, Bienstock RJ, Zeviani M, and Copeland WC (2004) Structure-function defects of human mitochondrial DNA polymerase in autosomal dominant progressive external ophthalmoplegia. Nat Struct Mol Biol 11, 770–776 [PubMed: 15258572]

48. Sowers ML, Anderson APP, Wrabl JO, and Yin YW (2019) Networked Communication between Polymerase and Exonuclease Active Sites in Human Mitochondrial DNA Polymerase. J Am Chem Soc 141, 10821–10829 [PubMed: 31251605]

49. Bratic A, Kauppila TE, Macao B, Grönke S, Siibak T, Stewart JB, Baggio F, Dols J, Partridge L, Falkenberg M, Wredenberg A, and Larsson NG (2015) Complementation between polymerase- and exonuclease-deficient mitochondrial DNA polymerase mutants in genomically engineered flies. Nat Commun 6, 8808 [PubMed: 26554610]

50. Nurminen A, Farnum GA, and Kaguni LS (2017) Pathogenicity in POLG syndromes: DNA polymerase gamma pathogenicity prediction server and database. BBA Clin 7, 147–156 [PubMed: 28480171]

51. Davidzon G, Greene P, Mancuso M, Klos KJ, Ahlskog JE, Hirano M, and DiMauro S (2006) Early-onset familial parkinsonism due to POLG mutations. Ann Neurol 59, 859–862 [PubMed: 16634032]

52. Euro L, Farnum GA, Palin E, Suomalainen A, and Kaguni LS (2011) Clustering of Alpers disease mutations and catalytic defects in biochemical variants reveal new features of molecular mechanism of the human mitochondrial replicase, Pol γ. Nucleic Acids Res 39, 9072–9084 [PubMed: 21824913]

53. Fujii T, Takase KI, Honda H, Kawamura N, Yamasaki R, Urata M, Uchiumi T, Iwaki T, and Kira JI (2019) Toxic myopathy with multiple deletions in mitochondrial DNA associated with long-term

use of oral anti-viral drugs for hepatitis B: A case study. Neuropathology 39, 162–167 [PubMed: 30847961]

54. Li M, Foli Y, Liu Z, Wang G, Hu Y, Lu Q, Selvaraj S, Lam W, and Paintsil E (2017) High frequency of mitochondrial DNA mutations in HIV-infected treatment-experienced individuals. HIV Med 18, 45–55 [PubMed: 27328746]
55. Nadalutti CA, Stefanick DF, Zhao ML, Horton JK, Prasad R, Brooks AM, Griffith JD, and Wilson SH (2020) Mitochondrial dysfunction and DNA damage accompany enhanced levels of formaldehyde in cultured primary human fibroblasts. Sci Rep 10, 5575 [PubMed: 32221313]
56. Naeem MM, Maheshan R, Costford SR, Wahedi A, Trajkovski M, Plavec J, Yatsunyk LA, Ciesielski GL, Kaufman BA, and Sondheimer N (2019) G-Quadruplex Mediated Reduction of a Pathogenic Mitochondrial Heteroplasmy. Hum Mol Genet
57. Lee Y, Kim T, Lee M, So S, Karagozlu MZ, Seo GH, Choi IH, Lee PCW, Kim CJ, Kang E, and Lee BH (2021) De Novo Development of mtDNA Deletion Due to Decreased POLG and SSBP1 Expression in Humans. Genes (Basel) 12
58. Belmonte FR, Martin JL, Frescura K, Damas J, Pereira F, Tarnopolsky MA, and Kaufman BA (2016) Digital PCR methods improve detection sensitivity and measurement precision of low abundance mtDNA deletions. Sci Rep 6, 25186 [PubMed: 27122135]
59. Lewis W, Copeland WC, and Day BJ (2001) Mitochondrial dna depletion, oxidative stress, and mutation: mechanisms of dysfunction from nucleoside reverse transcriptase inhibitors. Lab Invest 81, 777–790 [PubMed: 11406640]
60. Gandhi VV, and Samuels DC (2011) A review comparing deoxyribonucleoside triphosphate (dNTP) concentrations in the mitochondrial and cytoplasmic compartments of normal and transformed cells. Nucleosides Nucleotides Nucleic Acids 30, 317–339 [PubMed: 21774628]
61. Mandel H, Szargel R, Labay V, Elpeleg O, Saada A, Shalata A, Anbinder Y, Berkowitz D, Hartman C, Barak M, Eriksson S, and Cohen N (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat Genet 29, 337–341 [PubMed: 11687800]
62. Saada A, Shaag A, Mandel H, Nevo Y, Eriksson S, and Elpeleg O (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. Nat Genet 29, 342–344 [PubMed: 11687801]
63. Torregrosa-Muñumer R, Forslund JME, Goffart S, Pfeiffer A, Stojkovič G, Carvalho G, Al-Furoukh N, Blanco L, Wanrooij S, and Pohjoismäki JLO (2017) PrimPol is required for replication reinitiation after mtDNA damage. Proc Natl Acad Sci U S A 114, 11398–11403 [PubMed: 29073063]
64. Duong VN, Zhou L, Martínez-Jiménez MI, He L, Cosme M, Blanco L, Paintsil E, and Anderson KS (2020) Identifying the role of PrimPol in TDF-induced toxicity and implications of its loss of function mutation in an HIV+ patient. Sci Rep 10, 9343 [PubMed: 32518272]
65. Monnot S, Samuels DC, Hesters L, Frydman N, Gigarel N, Burlet P, Kerbrat V, Lamazou F, Frydman R, Benachi A, Feingold J, Rotig A, Munnich A, Bonnefont JP, and Steffann J (2013) Mutation dependance of the mitochondrial DNA copy number in the first stages of human embryogenesis. Hum Mol Genet 22, 1867–1872 [PubMed: 23390135]
66. Lee HC, Yin PH, Lu CY, Chi CW, and Wei YH (2000) Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem J 348 Pt 2, 425–432 [PubMed: 10816438]
67. Feng JY, Johnson AA, Johnson KA, and Anderson KS (2001) Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. J Biol Chem 276, 23832–23837 [PubMed: 11328813]
68. Foury F, and Szczepanowska K (2011) Antimutator alleles of yeast DNA polymerase gamma modulate the balance between DNA synthesis and excision. PLoS One 6, e27847 [PubMed: 22114710]
69. Reha-Krantz LJ (1998) Regulation of DNA polymerase exonucleolytic proofreading activity: studies of bacteriophage T4 “antimutator” DNA polymerases. Genetics 148, 1551–1557 [PubMed: 9560374]

70. Kasiviswanathan R, Longley MJ, Chan SS, and Copeland WC (2009) Disease mutations in the human mitochondrial DNA polymerase thumb subdomain impart severe defects in mitochondrial DNA replication. J Biol Chem 284, 19501–19510 [PubMed: 19478085]

71. Singh K, and Modak MJ (2005) Contribution of polar residues of the J-helix in the 3'−5' exonuclease activity of Escherichia coli DNA polymerase I (Klenow fragment): Q677 regulates the removal of terminal mismatch. Biochemistry 44, 8101–8110 [PubMed: 15924429]

72. Minnick DT, Bebenek K, Osheroff WP, Turner RM, Astatke M, Liu L, Kunkel TA, and Joyce CM (1999) Side chains that influence fidelity at the polymerase active site of Escherichia coli DNA polymerase I (Klenow fragment). J Biol Chem 274, 3067–3075 [PubMed: 9915846]

73. Luo N, and Kaguni LS (2005) Mutations in the spacer region of Drosophila mitochondrial DNA polymerase affect DNA binding, processivity, and the balance between Pol and Exo function. J Biol Chem 280, 2491–2497 [PubMed: 15537632]

74. He Q, Shumate CK, White MA, Molineux IJ, and Yin YW (2013) Exonuclease of human DNA polymerase gamma disengages its strand displacement function. Mitochondrion 13, 592–601 [PubMed: 23993955]

75. Beechem JM, Otto MR, Bloom LB, Eritja R, Reha-Krantz LJ, and Goodman MF (1998) Exonuclease-polymerase active site partitioning of primer-template DNA strands and equilibrium Mg2+ binding properties of bacteriophage T4 DNA polymerase. Biochemistry 37, 10144–10155 [PubMed: 9665720]

76. Macao B, Uhler JP, Siibak T, Zhu X, Shi Y, Sheng W, Olsson M, Stewart JB, Gustafsson CM, and Falkenberg M (2015) The exonuclease activity of DNA polymerase γ is required for ligation during mitochondrial DNA replication. Nat Commun 6, 7303 [PubMed: 26095671]

77. Johnson AA, Tsai Y, Graves SW, and Johnson KA (2000) Human mitochondrial DNA polymerase holoenzyme: reconstitution and characterization. Biochemistry 39, 1702–1708 [PubMed: 10677218]

78. Gray H, and Wong TW (1992) Purification and identification of subunit structure of the human mitochondrial DNA polymerase. J Biol Chem 267, 5835–5841 [PubMed: 1556099]

79. Codo AC, Davanzo GG, Monteiro LB, de Souza GF, Muraro SP, Virgilio-da-Silva JV, Prodonoff JS, Carregari VC, de Biagi Junior CAO, Crunfli F, Jimenez Restrepo JL, Vendramini PH, Reis-de-Oliveira G, Bispo Dos Santos K, Toledo-Teixeira DA, Parise PL, Martini MC, Marques RE, Carmo HR, Borin A, Coimbra LD, Boldrini VO, Brunetti NS, Vieira AS, Mansour E, Ulaf RG, Bernardes AF, Nunes TA, Ribeiro LC, Palma AC, Agrela MV, Moretti ML, Sposito AC, Pereira FB, Velloso LA, Vinolo MAR, Damasio A, Proença-Módena JL, Carvalho RF, Mori MA, Martins-de-Souza D, Nakaya HI, Farias AS, and Moraes-Vieira PM (2020) Elevated Glucose Levels Favor SARS-CoV-2 Infection and Monocyte Response through a HIF-1α/Glycolysis-Dependent Axis. Cell Metab 32, 498–499 [PubMed: 32877692]

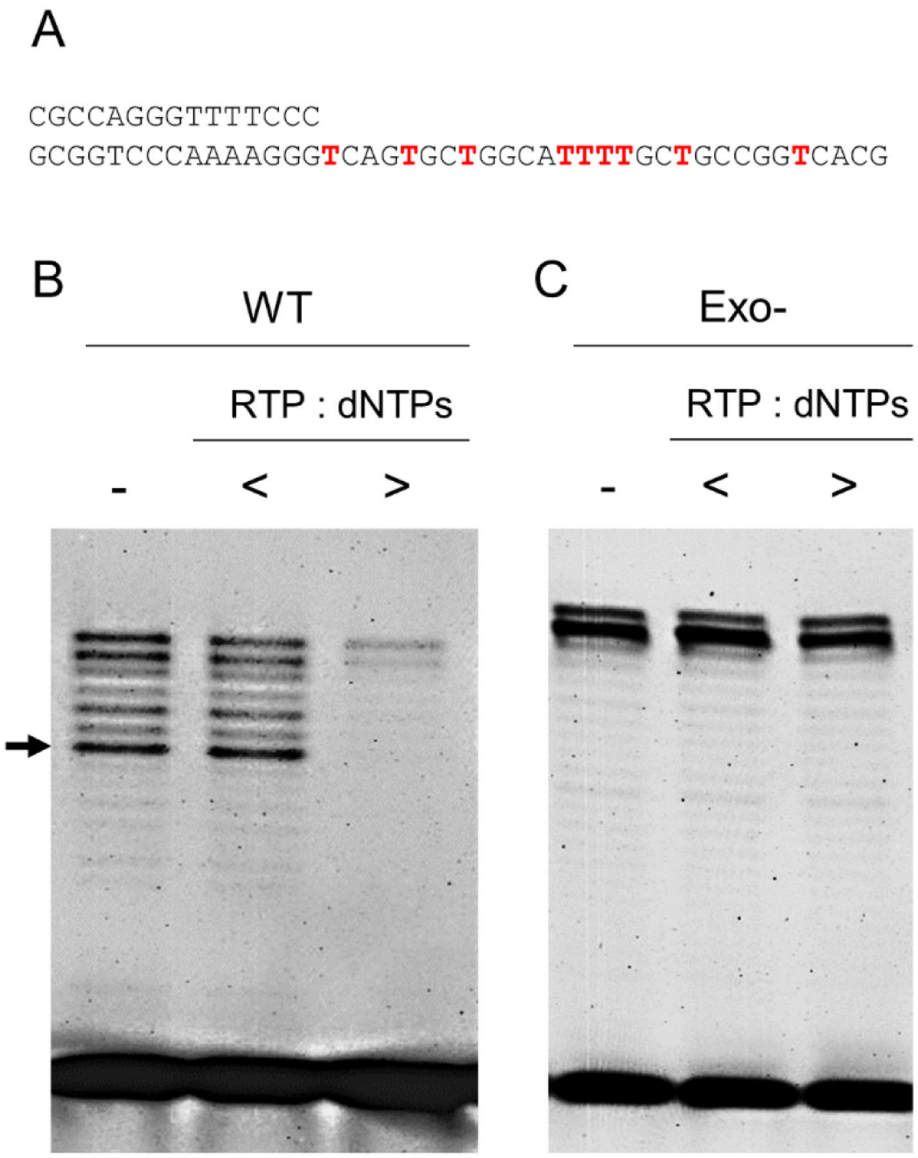

Figure 1. RTP inhibits DNA synthesis by Pol γ in vitro.

The primer extension assay was performed as described under "Materials and Methods", using a fluorescently-labeled primer-template (15/44) DNA substrate (A), and the wild-type (B) or the exonuclease-deficient (C) variant of Pol γ. The reactions were carried out in the presence of 0.8 mM dNTPs (0.2 mM each) and increasing concentrations of RTP: 0 (−), 0.2 (<) or 2 (>) mM, at 37°C for 20 minutes. The bold arrow in panel B indicates a product of exonucleolytic activity of the wild-type Pol γ (see also Supplementary Figure 1B). The gel images are representative of two independent analyses.

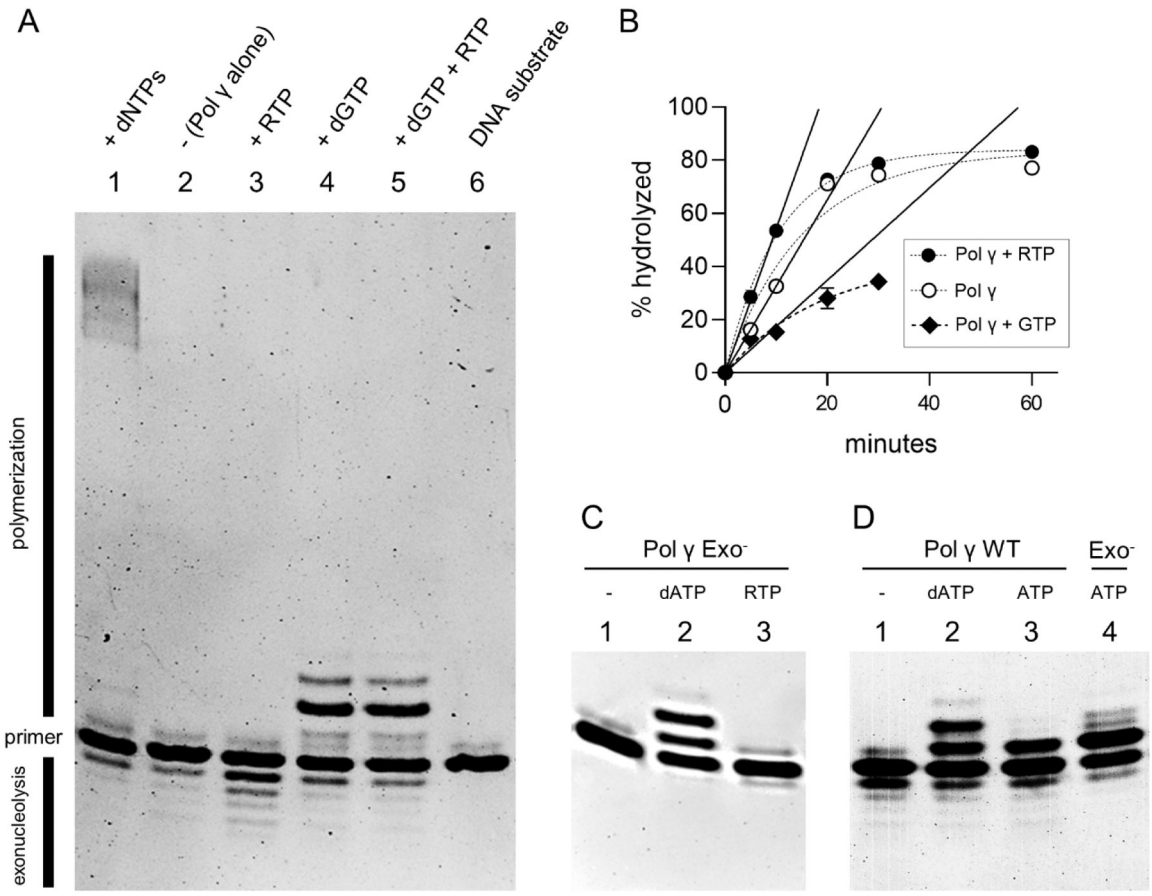

Figure 2. RTP stimulates the exonucleolytic activity of Pol γ.

A) The effects of RTP on the polymerase and exonuclease activities of Pol γ were analyzed by primer extension assay as described under "Materials and Methods". We compared the products of polymerization and/or exonucleolysis (labeled on the left) generated by the wild-type Pol γ in the presence or absence of 0.5 mM dNTPs, RTP, dGTP, or a combination of 0.5 mM RTP and 0.5 mM dGTP (as indicated), for 10 minutes at 37°C. B) The primer exonucleolysis time course experiment was performed as described under "Materials and Methods". The reactions were carried out using the wild-type Pol γ, alone (open circles), or in the presence of 0.5 mM RTP (closed circles), or 0.5 mM GTP (closed diamonds). The percent of hydrolysis represents the relative abundance of exonucleolysis products generated at the indicated time intervals (see also Supplementary Figure 2). Data points represent the mean of two experiments (+/− SD). Exonucleolysis rates were calculated from the slopes of linear regression fitted to the initial data points (straight lines), divided by the concentration of the enzyme in the reaction. C) The ability of Pol γ to incorporate RTP into a nascent DNA strand was inferred from the ability of Pol γ Exo$^-$ to extend the primer in the presence of 0.5 mM RTP (lane 3). As a control, the assay was performed in the absence of RTP (lane 1), or in the presence of 0.5 mM dATP as the incorporation reference (lane 2). Note that adenosine is the first nucleoside to be incorporated into the nascent strand (see Figure 1 A). D) To test how ribonucleotides affect the activity of Pol γ, the primer extension assay was carried out using the wild-type or Exo$^-$ Pol γ variant (as indicated), in the presence or

absence of 0.5 mM ATP (lanes 1,3,4). For the incorporation reference, the assay was carried out in the presence of 0.5 mM dATP (lane 2).

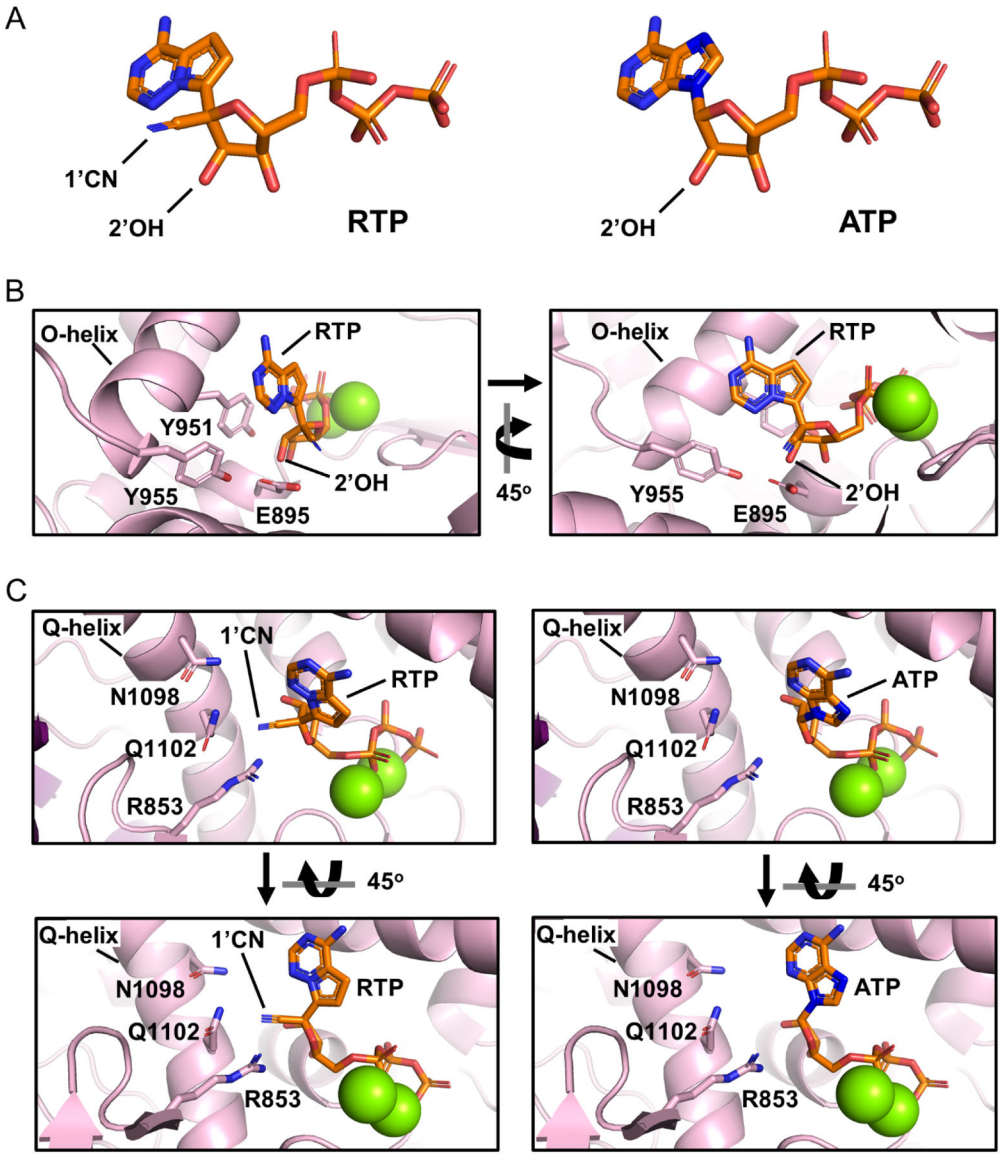

Figure 3. The cyano group (1'CN) of RTP may be sensed by Pol γ fidelity residues R853, N1098 and Q1102, triggering exonucleolysis.

A) Structures of RTP and ATP. B) Possible sensing of the 2' hydroxyl group (2'OH) of RTP by Pol γ residues E895, Y951 and Y955 in the pol catalytic site. C) Left panels, the 1'CN of RTP positions in close proximity to R853, N1098 and Q1102, simulating a distorted conformation of the primer-template similar to a nucleotide mismatch. Right panels, modeling of ATP in the pol catalytic site would not simulate a similar distortion. The catalytic Mg^{2+} ions are represented as green spheres

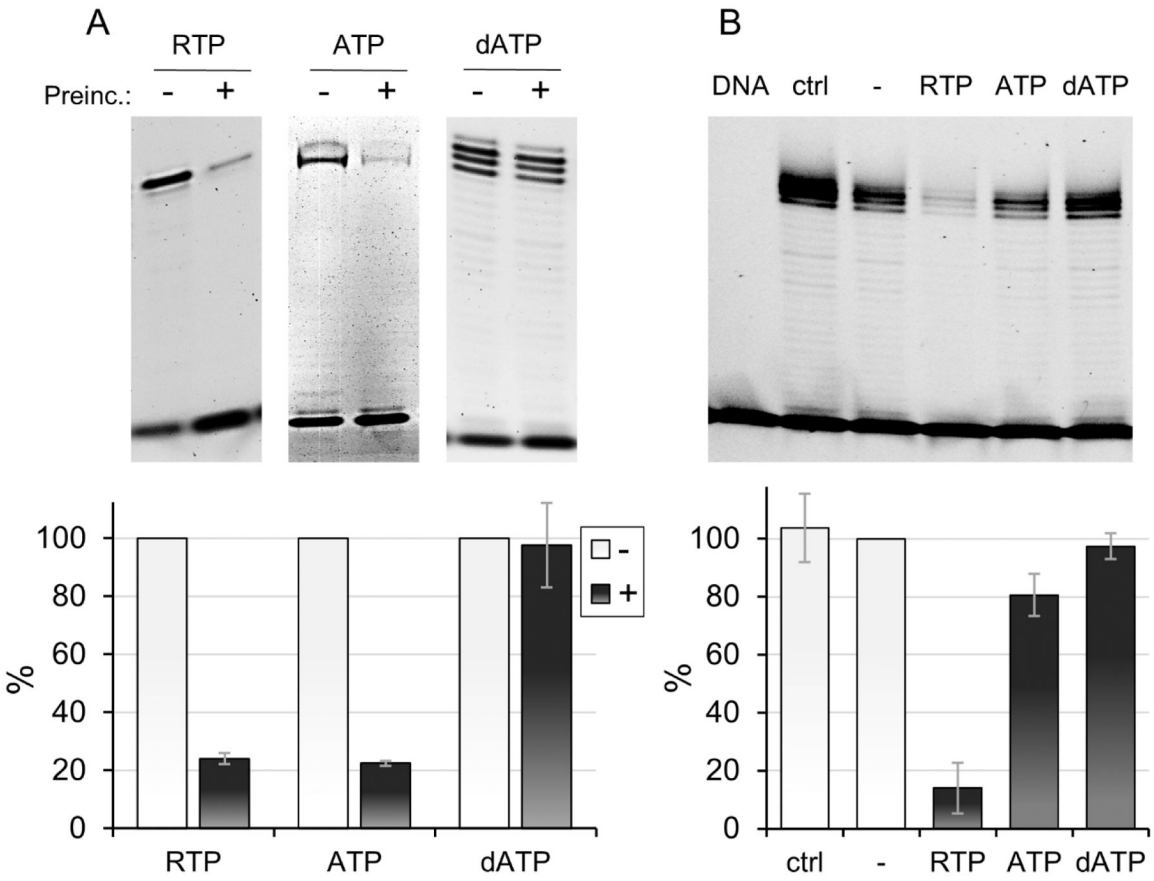

Figure 4. The inhibitory effect of RTP on the DNA synthesis activity of Pol γ is persistent.

A) The inhibitory effect of RTP and ATP on DNA synthesis by Pol γ cannot be reversed by excess nucleotides. The primer extension assay was performed as described under "Materials and Methods", using the wild-type Pol γ, 0.5 mM RTP, ATP, or dATP, and 10 mM (total) dNTPs. Pol γ was preincubated for 5 minutes at room temperature, in the presence (+) or absence (−) of RTP, ATP, or dATP (as indicated). The premix was then added to the reaction mixture containing excess dNTPs, as well as RTP, ATP, or dATP if these were absent in the preincubation step (−). The abundance of the full-length products was normalized to reactions performed in the absence of RTP or ATP in the preincubation step (−) and the means of two experiments (+/− SD) were presented on a graph (bottom panel). B) The capacity of Pol γ to 'recover' after inhibition by RTP or ATP and reinitiate DNA synthesis on a new DNA substrate was tested by a staged primer extension assay, as described under "Materials and Methods". Briefly, the wild-type Pol γ was initially incubated in the reaction mixture containing an unlabeled DNA substrate, in the presence (+) or absence (−) of 0.5 mM RTP, ATP, or dATP (as indicated) at 37°C. After 10 minutes, four-fold excess of a second, fluorescently-labeled DNA substrate was added to the reaction mixture together with 2 mM dNTPs, and the incubation continued for the next 20 minutes. As the control for the maximal DNA synthesis efficiency (ctrl), we performed the reaction in the absence of the primary unlabeled substrate and RTP, ATP, or dATP, with no preincubation step, for 20 minutes at 37°C (essentially an uninhibited primer extension over the labeled DNA substrate). The abundance of the full-length products was normalized to

reactions performed in the absence of RTP, ATP, or dATP (−) and the means of at least three experiments (+/− SD) were presented on a graph (bottom panel).

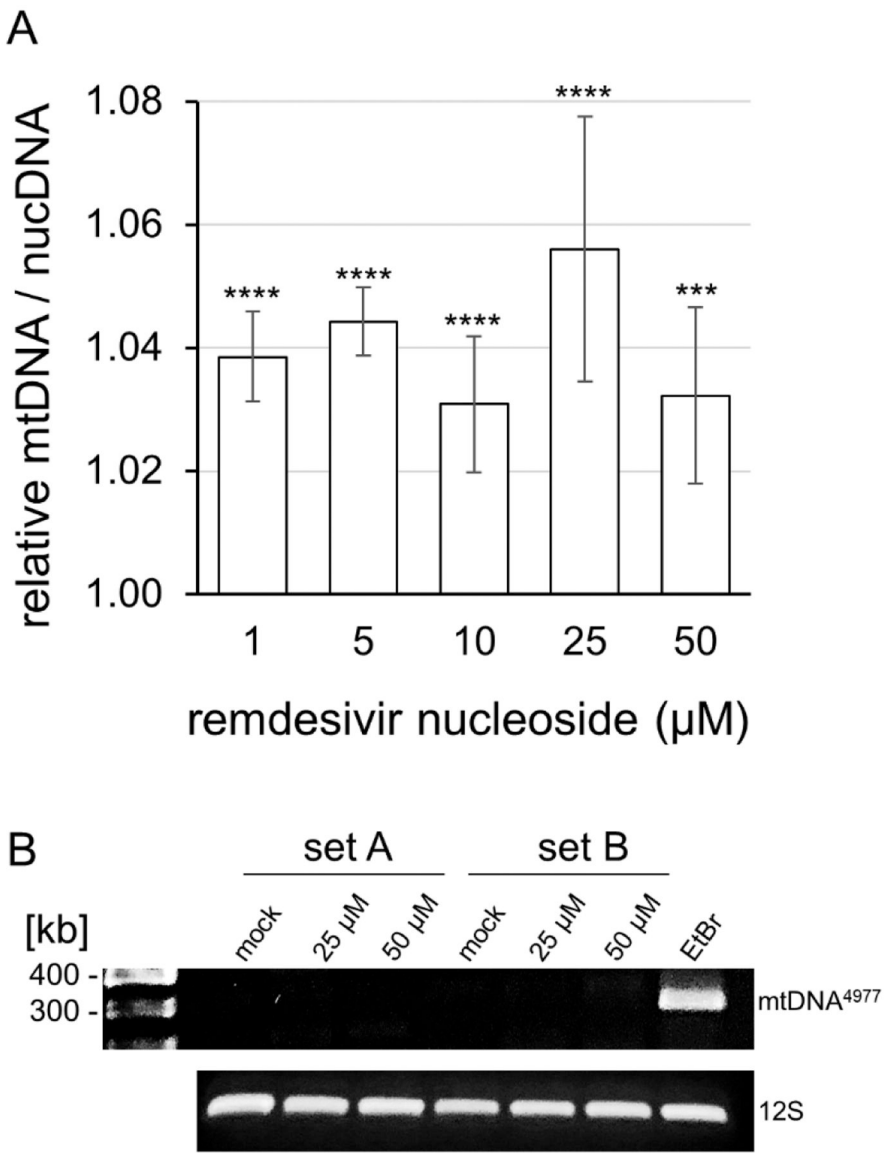

Figure 5. Human neonatal dermal fibroblasts exhibit increased mtDNA copy number and no presence of the common deletion upon remdesivir nucleoside treatment.

Fibroblasts were cultured for three days after which remdesivir nucleoside (GS-441524) was added to the media to the indicated final concentration. Cells were collected after seven days of treatment. A) Total DNA was isolated and the relative mtDNA copy number was analyzed by Q-RT-PCR, as described under "Materials and Methods". The data represent means of triplicates of two biological repeats, normalized to untreated cells (+/− SD). The statistical analysis was done by one-way ANOVA test followed by post-hoc Dunnett's comparison of the remdesivir nucleoside-treated cells to the untreated cells, in GraphPad Prism. *****P*<0.0001, ***P<0.001. B) The presence of the common deletion in mtDNA ($mtDNA^{4977}$) in cells treated with the indicated concentration of remdesivir nucleoside was tested by nested PCR, as described in the "Materials and Methods". The presence of mtDNA was confirmed by amplification of the 12S mitochondrial gene (12S). For the positive control of the common deletion induction, fibroblasts were treated with 50 μM ethidium

bromide (EtBr) as described in the “Materials and Methods”. The presence of the common deletion in the PCR product was confirmed by sequencing.