jz-2022-01314x.R1

Name: Peer Review Information for "Unveiling the “Template-dependent” Inhibition on the Viral Transcription of SARS-CoV-2"

First Round of Reviewer Comments

Reviewer: 1

Comments to the Author

The authors present a simulation study of the template-dependent inhibition mechanism of Remdesivir. The results are interesting since it reveals a potentially novel mechanism. There are a few minor questions that need to be answered below:

1) Line 18 on page 2, what is the full name of the NTP?

2) Line 5 on page 6, the ms presents that five 50 ns simulations have been performed and even extended to 100 ns. Why could the short 100ns simulations demonstrate the stability of such large complexes? This should be clarified further.

3) Line 55 on page 6, the ms presents that the previous modeling has suggested the cyano group of Remdesivr at the +1 site clashed with the backbone of the A588. However, the authors observed that A558 moved away from it. Which crystal structure (PDB) was used in the previous simulation? Is the initial conformation different from the current one in the ms? Fig.S2 only presents the static distance distributions and snapshots. It is better to show the simulated conformational change of A558 with time.

4) Line 53 on page 8, "a translocation pathway was generated by the Climber algorithm". No structural information is provided in Section 3 of SI. A few snapshots of the intermediates are needed to be shown.

5) In Figures 2A, 2C, and 2E, the sites such as "+1" needed to be labeled.

6) Line 17 on page 12, "at the unprecedented molecular level"? Maybe the authors mean that an unprecedented mechanism at the molecular level?

Reviewer: 2

Comments to the Author

Title: Unveiling the “template-dependent” inhibition on the viral transcription of SARS-CoV-2

Luo, Wang, Yao, Gao, and Zhang

Summary:

In this research report, the authors use molecular simulation to investigate a second inhibition mechanism of remdesivir against SARS-CoV-2 RNA-dependent RNA polymerase (RdRp). Experiments by the Matthias Götte group have demonstrated that in addition to the "delayed chain termination" exerted by remdesivir after incorporation into the primer strand of RNA, remdesivir also inhibits RNA replication after incorporation in the template strand by interfering with polymerization of its complement nucleotide. The authors investigate this phenomenon through a variety of molecular dynamics (MD) simulations using two of the available cryo-EM structures for SARS-CoV-2 as starting points for models with normal RNA and wild-type RdRp, Remdesivir in the template strand at various positions, RdRp with a V557L, and intermediate structures of Remdesivir between the +2 and +1 position.

Major Comments:

In this work the authors correctly pursue a biologically relevant mechanism that is not as well understood for nucleoside analogs as chain termination—template-dependent inhibition. This appears to be especially important for remdesivir since the delayed chain termination it achieves can be overcome with an increased concentration of nucleotides, essentially pushing the equilibrium of the reaction to the polymerized product. A second chance for remdesivir comes when its RNA strand becomes the template and it interferes with the catalysis of UTP in the primer strand opposite its position. The MD simulations performed here help to address the molecular basis of this mechanism, but I am asking for major changes in the manuscript to make it suitable for publication.

• The introduction needs reworking to make it clear why the template-dependent inhibition is important and how the MDs are suitable tools to investigate this effect. How much of remdesivir's antiviral activity is due to this instead of delayed chain termination? What can we expect to learn by the series of MD simulations?

• The model building process needs to be expanded. This can continue to be in the supplementary methods, but there are major modifications happening to the cryo-EM structures in order for the simulations to be at the desired starting point, the precatalytic state. For example, the 7BZF structure has remdesivir incorporated into primer strand and translocated to the -1 position. Why was this structure used instead of 7BV2 which has remdesivir incorporated but not translocated? How did the structure change after the NTP and Mg cations were added from the Norovirus RdRp and the structure minimized? Is your approach sensitive to differences in starting positions?

• The discussion would greatly benefit from better integration with the published experimental and modeling results. For example, modeling done by Tchesnokov et al. 2020 suggested that remdesivir is a poor template for the incoming UTP because base pairing is compromised. Yet your simulation predicted that base pairing is stable. Does the protein backbone require large shifts to prevent clashing with A558? Is there experimental evidence for the prediction that inhibition is due to +2 to +1 translocation versus Tchesnokov's proposal of compromised base-pairing? I understand the translocation pathway saw a difference between wild-type RdRp and V557L, but how do we know the conformation in Fig 3G is not just an artifact of a high-energy contact as remdesivir is translocated.

Minor Comments:

• The manuscript would benefit from copyediting, ie. "health crisis" vs. "healthy crisis."

• The figures could be greatly enhanced with better labeling and 3D structures. For example, figure 2 could use structures to illustrate the measurements like in figure 4.

Author's Response to Peer Review Comments:

Dear Prof Editor,

Thank you for forwarding us the editorial and reviewers' comments on our manuscript entitled: "Unveiling the "Template-dependent" Inhibition on the Viral Transcription of SARS-CoV-2" with the manuscript ID: jz-2022-01314x. We are writing to submit our revised manuscript.

We appreciate the editorial instructions and thank both reviewers for their thoughtful comments. During this revision, we have performed extra ~2 μs MD simulations to address the reviewers' comments. Below we have attached our point-to-point responses to the comments and described how we have modified the manuscript accordingly. All the changes have been highlighted in red in both the revised maintext and SI. We hope that these modifications make the paper fit for the publication. Again, we appreciate the helpful instructions and insightful comments raised by the editor and reviewers, and we will be happy to elaborate more if necessary.

Sincerely yours,

Lu Zhang
Professor
State Key Laboratory of Structural Chemistry, Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, Fuzhou, Fujian, China
University of Chinese Academy of Sciences, Beijing, China
E-mail: luzhang@fjirsm.ac.cn

Response to Editorial Comments:

1.Title: In both the main manuscript file and the Supporting Information, set the title in title case, with the first letter of each principal word capitalized.

Reply: We would like to thank the editor for the instruction. Accordingly, we have capitalized the first letter of each principal word in the title in both the revised maintext and the revised Supporting Information.

2.Title: Using acronyms in title is discouraged. Please spell out all acronyms in the title of the manuscript and Supporting Information.

Reply: We have contacted the editorial office before about this comment and followed the instruction to keep the acronym "SARS-CoV-2" as it is in the title.

3. Abstract: Shorten the abstract to 150 words or fewer. Your abstract is currently 174 words.

Reply: In this revision, we have shortened the abstract to 146 words.

4.TOC Graphic: Provide a TOC image per journal guidelines (2 in x 2 in; on the same page as the abstract) with the heading "TOC Graphic" above the graphic. The graphic should be in the form of a structure, graph, drawing, photograph, or scheme—or a combination. Non-scientific cartoon-like images or caricatures are discouraged.

Reply: In the revised manuscript, the following TOC image (Fig. R1) per journal guidelines has been inserted on the same page as the abstract.

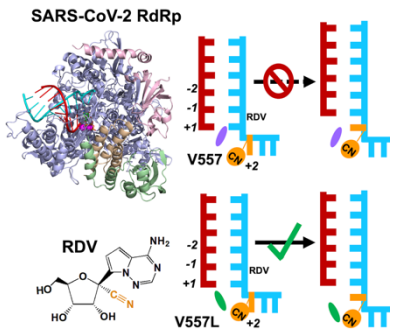

Fig. R1 TOC graphic (2inch x 2inch)

5.References: In both the main file and the supporting information, fix the style of all references to use JPCL formatting (check all references carefully). ***JPC Letters reference formatting requires that journal references should contain: () around numbers, author names, article title (titles entirely in title case or entirely in lower case), abbreviated journal title (italicized), year (bolded), volume (italicized), and pages (first-last). Book references should contain author names, book title (in the same pattern), publisher, city, and year.

Reply: Thanks for the detailed instruction about the reference style. We have accordingly checked/fixed the reference style in both the maintext and supporting information to be compatible with the JPCL formatting.

6. Author Affiliations: Please include postal codes/country in the author affiliations in the publication file(s).

Reply: We have inserted the postal codes in the author affiliations.

7. Supporting Information: Please number SI pages in the following format: "S1, S2…"

Reply: In the revised Supporting Information, the SI pages have been numbered following the required format.

8. Please include annotated version(s) of your revised publication file(s) with colored text or highlights indicating the revisions that you have made, and upload them as "Supporting Information for Review Only." Please also upload "clean" copies for publication. (No highlighting, annotations, or colored text.)

Reply: We have uploaded the revised maintext and SI with the changes highlighted in red as "Supporting Information for Review Only". The copies without mark have been uploaded as "Manuscript file" and "Supporting Information for publication".

Response to Reviewer#1's Comments:

The authors present a simulation study of the template-dependent inhibition mechanism of Remdesivir. The results are interesting since it reveals a potentially novel mechanism. There are a few minor questions that need to be answered below.

Reply: We would like to thank this reviewer to point out that our results are interesting and have revealed a potentially novel template-dependent mechanism of Remdesivir. However, he/she also raised a few minor questions for us to improve our manuscript. Please see below for our point-to-point response to his/her comments.

1. Line 18 on page 2, what is the full name of the NTP?

Reply: We would like to thank the reviewer for pointing this out and the full name of NTP is nucleoside triphosphate. In the revised maintext, we have edited the sentence as follows to insert the full name:

"*… three nucleoside triphosphates (NTPs) can be …*"

2. Line 5 on page 6, the ms presents that five 50 ns simulations have been performed and even extended to 100 ns. Why could the short 100ns simulations demonstrate the stability of such large complexes? This should be clarified further.

Reply: We would like to thank the reviewer for this good comment. We have followed the suggestion of the reviewer and examined the stability of the complex. In specific, we aligned all the MD conformations to the seed conformation based on the 926 C_α atoms of nsp12 and then computed the root mean square root (RMSD) of the C_α atoms (Fig. R2). We have found out that the 100ns simulations have been well equilibrated as the RMSD values have reached plateau at around 10ns, which also validates the stability of the complex.

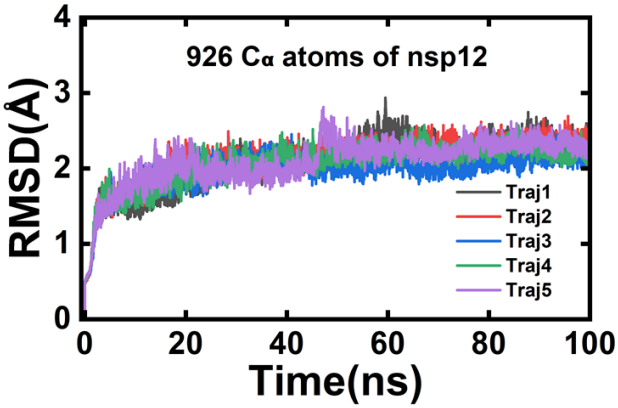

Fig. R2 The RMSD of C_α atoms of nsp12 versus time in the five 100ns MD simulations. All the 926 C_α atoms in nsp12 were used for the structural alignment and RMSD calculations.

To further validate the convergence of the simulations for the current study, in this revision we have extended each of the five simulations to 200ns for both the "wildtype RNA" system as well as the system with Remdesivir at *+1* site of template strand ("RDV at *+1* site" system). In this regard, for each system, we have MD conformations from accumulated 1μs simulations for examining the stability of active site (Fig. R3). In specific, we have first used the MD conformations from 5×50ns, 5×100ns, 5×150ns

and 5×200ns simulations to compute the P_α-O3' distance, which is critical for phosphodiester bond formation during NTP incorporation (Fig. R3A). Our results have shown that in both systems, the catalytically active conformations are well maintained and the P_α-O3' distances are similar when computed using simulations of different lengths (Fig. R3A-R3C). Moreover, we have found out both of the systems could sustain the base pairing and base stacking stability at the active site when extending the simulations up to 200ns, as the hydrogen bonding probability for base pair at the active site and the base stacking angle computed from 5×100ns, 5×150ns and 5×200ns (light purple bar in Fig. R3D-R3I) resemble that computed using 5×50ns simulations (green bar in Fig. R3D-R3I). These observations have altogether consolidated that our simulations are sufficiently long to examine the stability of the active site in the precatalytic state of SARS-CoV-2 RdRp.

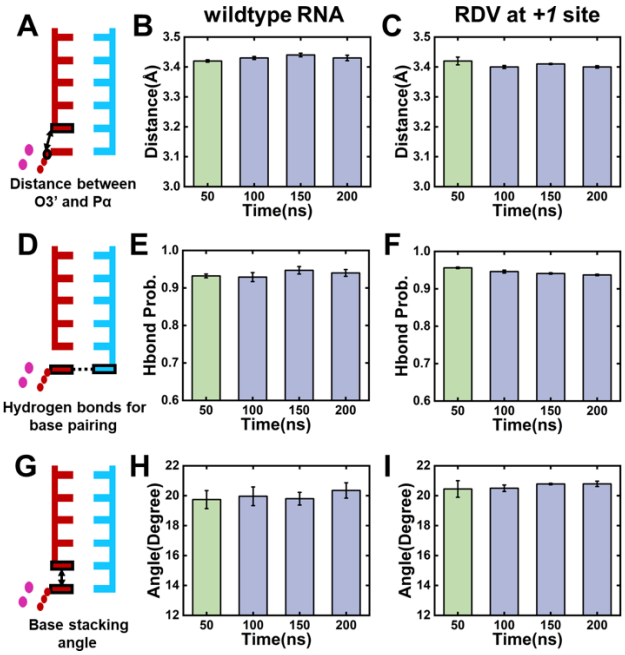

Fig. R3 Validation of the convergence of the simulations. (A) A cartoon showing the distance between P_α atom of NTP and the O3' atom of the 3'-terminal nucleotide in the nascent strand. (B) The P_α-O3' distance calculated using 5×50ns (in green), 5×100ns, 5×150ns and 5×200ns (in light purple) simulations for RdRp with wildtype RNA system ("wildtype RNA"). (C) Similar to (B) but for the RdRp with Remdesivir (RDV) embedded at *+1* site of the template strand ("RDV at *+1* site"). (D) A cartoon showing the hydrogen bonds for base pair at the active site. (E) Hydrogen bonding probability for the UTP:A pair at the active site in the "wildtype RNA" system calculated using 5×50ns (in green), 5×100ns, 5×150ns and 5×200ns (in light purple) simulations. (F) Similar to (E) but for the UTP:RDV pair at the active site in the "RDV at *+1* site" system. (G) A cartoon showing the base stacking between UTP and the 3'-terminal nucleotide in the nascent strand. (H) The base stacking angle computed using 5×50ns (in green), 5×100ns, 5×150ns and 5×200ns (in light purple) simulations for the "wildtype RNA" system. (I) Similar to (H) but for the "RDV at *+1* site" system.

Again, we would like to thank the reviewer for this great comment and accordingly, we have replaced the original Fig. S1 with Fig. R3 in the revised SI. Besides, we have edited the following sentences to include the above discussions in the revised maintext:

"*…we have extended each of the five replicas of 50ns simulations to 200ns and found out that the capability of UTP in maintaining the catalytically active conformation at the active site computed using 5×100ns, 5×150ns or 5×200ns MD simulations is similar to that as observed in 5×50ns simulations (Fig. S1). Moreover, we have also computed the root mean square deviations (RMSD) of the C_α atoms in the nsp12 along the MD trajectories and found out that the RdRp is stable in our simulations (Fig. S2). …*"

To ensure the compatibility, we have further computed the RMSD of the C_α atoms in the nsp12 using the 5×200ns simulations and inserted this figure as Fig. S2 in the revised SI.

3. Line 55 on page 6, the ms presents that the previous modeling has suggested the cyano group of Remdesivr at the +1 site clashed with the backbone of the A588. However, the authors observed that A558 moved away from it.

Reply: In the previous work, they have constructed a model and observed that A558 would clash with Remdesivir at *+1* site of the template strand and move away from Remdesivir. In our work, we have performed MD simulations and also consistently observed that A558 stays apart from Remdesivir due to the steric clash. In the revised maintext, we have edited the sentence as follows to clarify this point:

"*… a previous study based on the structural modeling has suggested that the backbone of the protein residue A558 would clash with the 1'-cyano group of Remdesivr at +1 site, thereby departing from Remdesivir and destabilizing the base pairing between UTP and Remdesivir. …*"

(1) Which crystal structure (PDB) was used in the previous simulation? Is the initial conformation different from the current one in the ms?

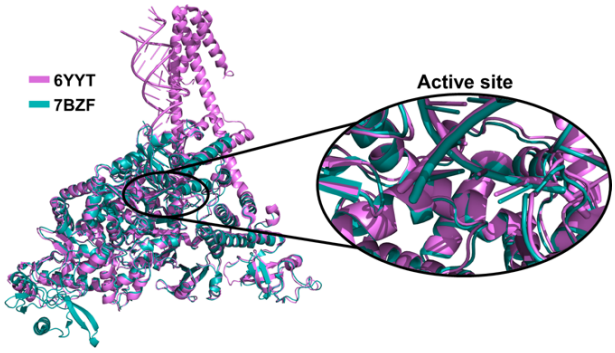

Fig. R4 Comparison of the two cryo-EM structures of SARS-CoV-2 RdRp. The structure with PDBID 6YYT is shown in violet and the other structure with PDBID 7BZF is shown in teal. The overall architecture of two RdRp structures are shown on the left while the configurations of the active site in two structures are amplified on the right.

Reply: The model set-up of the current work is different from that used in the previous modeling (*J. Biol. Chem.* 295, 16156-16165 (2020)). In the previous work, they used the cryo-EM structure of SARS-CoV-2 RdRp complex (PDBID: 6YYT, shown in violet in Fig. R4) as the basis to construct the model, with a further structural alignment to the X-ray structure of HCV RdRp (PDBID: 4WTD) to obtain the NTP and Mg^{2+} ions.

In our model set-up, we have used another cryo-EM structure of SARS-CoV-2 RdRp complex (PDBID: 7BZF, shown in teal in Fig. R4) and modelled the NTP and Mg^{2+} ions in the active site by aligning to the Norovirus RdRp (PDBID:3H5Y). Although the structural basis used in the current work and the previous work are originated from different publications, both cryo-EM structures demonstrate high conformational similarity in the core region of RdRp complex (Fig. R4). We also noticed that the cryo-EM with PDBID 6YYT has an extra turn of RNA in the upstream region and longer nsp18 extensions in comparison with the other cryo-EM structure (PDBID: 7BZF). However, these structural components are not in contact with the active site region, and thus would not exert obvious influence on the current investigations around the active site.

To further examine if different initial conformations would lead to different observations in the active site, we have constructed two extra RdRp models (named as "6YYT-4WTD" and "7BZF-4WTD") and performed MD simulations (see the response to comment #2(2) of Reviewer #2 for details). Our results have demonstrated that in both the new models and our current model, A558 would move away when Remdesivir is present at *+1* site of the template strand, leading to a larger separation distance than that when adenosine is located at *+1* site of the template strand (Fig. R5). These observations have suggested that even though different experimental structures were used for the model construction, A558 would always be repelled away from Remdesivir due to the steric clash exerted by the 1'-cyano substitution.

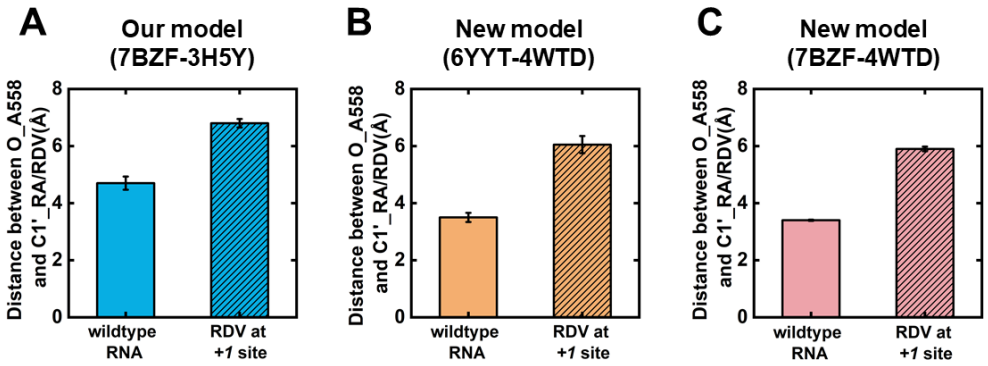

Fig. R5 The distance between A558 and adenosine/Remdesivir at *+1* site of the template strand in the MD simulations initiated from different models. For both "wildtype RNA" and "RDV at *+1* site" systems initiated from each model, 5×50ns MD simulations were performed to estimate the mean values and standard deviations of the distances.

In the revision, we have added Fig. R5B-R5C as Fig. S5D in the revised SI. Besides, we have inserted the following sentences to include the above discussions in the revised maintext:

"*… We also noted that the previous study has used an alternative cryo-EM structure of SARS-CoV-2 RdRp (PDBID: 6YYT) as the basis to construct the model. To further examine if the discrepancy of the base pairing stability is originated from the modelling using different experimental structures, we have constructed two extra models by referring to the previous study. MD simulations have been performed to examine the stability of the active site as well as the position of A558 relative to the template strand*

nucleotide (Fig. S5, see SI Section 4 for details). We have found out that even though different experimental structures were used for the initial modelling, the UTP: Remdesivir base pair at the active site is always well maintained, and A558 would move away from Remdesivir, further consolidating the robustness of our results. …"

(2) Fig.S2 only presents the static distance distributions and snapshots. It is better to show the simulated conformational change of A558 with time.

Reply: We would like to thank the reviewer for this great suggestion. When Remdesivir is present at *+1* site of the template strand, it repels A558 away with a larger inter-molecular distance after the energy minimization (EM) and such a separation between A558 and Remdesivir is well maintained in the whole simulations (Fig. R6). In specific, the distance between the backbone oxygen of A558 and C1' of Remdesivir at *+1* site of template strand is ~6.9 Å after energy minimization and such a distance is further relaxed to stay around 6.5 Å in the MD simulations. To clearly demonstrate the conformations of A558 with time, we have also shown the energy minimized conformation as well as the MD snapshots at 20ns, 40ns, 60ns, 80ns and 100ns (inset in Fig. R6). As a comparison, we have also shown the energy minimized conformation of the "wildtype RNA" system (display in white in the inset of Fig. R6). It is obvious that A558 is farther away from Remdesivir at *+1* site of template strand than that from adenosine at the respective site, not only in the energy minimized conformation, but also in the MD conformations.

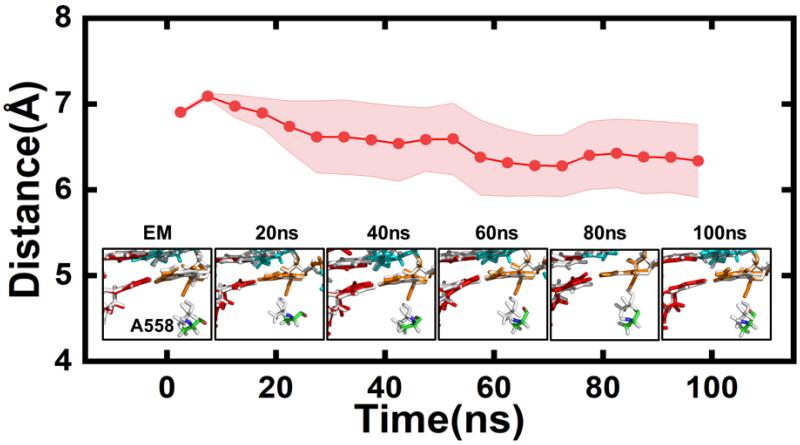

Fig. R6 The conformations of A558 along the MD simulations. The red line with circles denotes the distance between A558 and the C1' atom of Remdesivir at *+1* site of template strand along the MD trajectories. Each symbol labels the mean distance averaged by five trajectories using 5ns as a window. The transparent red background represents the standard deviations. The energy minimized conformation as well as the MD snapshots at 20ns, 40ns, 60ns, 80ns and 100ns from one random trajectory of the "RDV at *+1* site" system are shown as inset, where the energy minimized conformation of the "wildtype RNA" system is shown in white for comparison.

In the revised SI, Fig. R6 has been inserted as Fig. S4, and cited in the following sentence in the revised maintext:

"*… A558 would move away from the 1'-cyano group of Remdesivir (Fig. S3 and S4). …*"

4. Line 53 on page 8, "a translocation pathway was generated by the Climber algorithm". No structural information is provided in Section 3 of SI. A few snapshots of the intermediates are needed to be shown.

Reply: We thank the reviewer for this great suggestion and accordingly we have demonstrated the snapshots of the intermediates along the translocation pathway (Fig. R7).

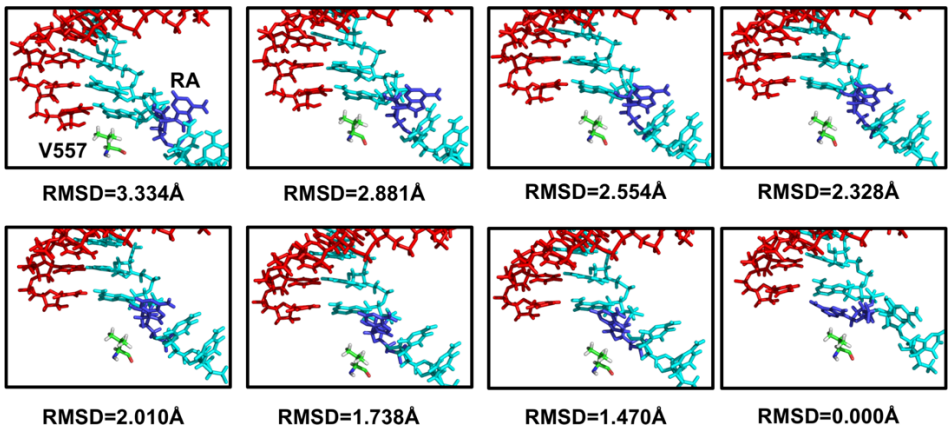

Fig. R7 The conformations along the preliminary translocation pathway generated by Climber algorithm. The adenosine (RA) translocating from *+2* to *+1* site is shown in blue. For each conformation, the RMSD relative to the conformation of the post-translocation state is shown below, and all the heavy atoms of nucleotides were included for the RMSD calculations.

In the revised SI, this Fig. R7 has been inserted as Fig. S7 and cited in the maintext as follows:

"*…, a translocation pathway was generated by the Climber algorithm (Fig. S7) …*"

5. In Figures 2A, 2C, and 2E, the sites such as "+1" needed to be labeled.

Reply: We have added the site labels "*+1*" and "*-1*" in Figs. 2B, 2E and 2H of the revised maintext (Figs. 2A, 2C and 2E of the original maintext).

6. Line 17 on page 12, "at the unprecedented molecular level"? Maybe the authors mean that an unprecedented mechanism at the molecular level?

Reply: We apologize for the inappropriate wording and in this revision, we have followed the reviewer's suggestion and edited this sentence as follows:

"*… but also elucidates an unprecedented mechanism at the molecular level. …*"

Response to Reviewer#2's Comments:

In this work the authors correctly pursue a biologically relevant mechanism that is not as well understood for nucleoside analogs as chain termination—template-dependent inhibition. This appears to be especially important for remdesivir since the delayed chain termination it achieves can be overcome with an increased concentration of nucleotides, essentially pushing the equilibrium of the reaction to the polymerized product. A second chance for remdesivir comes when its RNA strand becomes the template and it interferes with the catalysis of UTP in the primer strand opposite its position. The MD simulations performed here help to address the molecular basis of this mechanism, but I am asking for major changes in the manuscript to make it suitable for publication.

Reply: We thank the reviewer for pointing out that our work has correctly pursued a biologically relevant but not well understood mechanism—"template-dependent" inhibition, and our MD simulations have helped to address the molecular basis of this mechanism. However, he/she also raised some major comments for us to improve our manuscript, mainly about modifying the introduction and investigating the effect of initial modelling on the results. In this revision, we have carefully addressed his/her comments and our responses have been attached below.

1. The introduction needs reworking to make it clear why the template-dependent inhibition is important and how the MDs are suitable tools to investigate this effect. How much of remdesivir's antiviral activity is due to this instead of delayed chain termination? What can we expect to learn by the series of MD simulations?

Reply: We would like to thank the reviewer for this great comment. The "template-dependent" inhibition is an alternative mechanism exerted Remdesivir when it is present at the template strand in SARS-CoV-2 RdRp. Compared to the well study "delayed chain termination" acted by Remdesivir at the nascent strand, the "template-dependent" inhibition has been less understood. Investigation of such inhibitory mechanism is one important aspect for gaining a comprehensive understanding about the action of Remdesivir on SARS-CoV-2 RdRp. In specific, the "delayed chain termination" is obvious when the NTP concentration is ~ 0.1μM and only less than 10% of the full-length RNA product can be observed (*J. Biol. Chem.* 295, 16156-16165 (2020)). However, when higher concentrations of NTP pools were used, the RNA synthesis arrest caused by the "delayed chain termination" would be overcome and more than 90% full-length product can be yielded at the NTP concentration as low as 10 μM (Fig. 6B in *J. Biol. Chem.* 295, 6785-6797 (2020); Fig. 1C in *J. Biol. Chem.* 295, 16156-16165 (2020)). As the intracellular NTP concentrations are in the range of high μM and low mM (*Mol. Cell. Biochem.* 140, 1-22 (1994); *J. Biol. Chem.* 285, 39380-39391 (2010)), the efficient read-through likely occurs under biologically relevant conditions to generate full-length RNA product containing Remdesivir, which would be used as the template strand for the synthesis of the second RNA strand. Further biochemical experiment has shown that Remdesivir present at the template strand would reduce the NTP incorporation efficiency and an NTP concentration of 50~100 μM is required to overcome this obstacle and recover over 90% NTP incorporation

efficiency (Fig. 3B in *J. Biol. Chem.* 295, 16156-16165 (2020)). It is worthy to note that such a concentration is obviously higher than that required to bypass the "delayed chain termination", suggesting that the "template-dependent" inhibition is pronounced under biologically relevant condition. Therefore, "template-dependent" inhibition is important for the action of Remdesivir on SARS-CoV-2 RdRp, while the contribution of two mechanisms to the antiviral activity of Remdesivir would be highly dependent on the NTP concentrations. Besides, when multiple Remdesivir-triphosphates are incorporated into the nascent strand, the "delayed chain termination" could be amplified.

MD simulations have been a useful tool to monitor the dynamics of biomolecules and supplement the experiments to elucidate the underlying mechanisms at the molecular level. Since the outbreak of COVID-19 pandemic, MD simulations have been widely used to understand the acting mechanism of SARS-CoV-2 RdRp inhibitors as well as to explore the potential inhibitors (for example, *J. Phys. Chem. B.* 124, 6955-6962 (2020); *Mol. Syst. Des. Eng.* 6, 888-902 (2021); *Phys. Chem. Chem. Phys.* 23, 20117-20128 (2021); *J. Phys. Chem. Lett.* 13, 4111-4118 (2022)). In particular, the "delayed chain termination" mechanisms have been investigated by MD simulations (*Phys. Chem. Chem. Phys.* 23, 5852-5863 (2021); *ACS Cent. Sci.* 7, 164-174 (2021)), the results of which have not only well reproduced the experimental observations that the termination occurs at the upstream site of the nascent strand instead of the active site, but also further elucidated the underlying mechanisms. These previous works have suggested that MD simulations are useful in elucidating the inhibitory mechanisms of nucleoside analogs and thus serve as a promising tool for the current investigation of the "template-dependent" inhibition acted by Remdesivir in SARS-CoV-2 RdRp. For example, the ensemble of MD conformations could be used to estimate the thermal stability of UTP:Remdesivir pair at the active site under dynamic protein environment, the information of which in incapable to be provided by static experimental structures or models.

Moreover, besides understanding how Remdesivir inhibits its complementary UTP incorporation, our current work also focused on the mechanism about how the RdRp with V557L mutation gains the drug resistance to Remdesivir, the prerequisite of which is to explore the conformations of V557L. However, the available experimental structures of SARS-CoV-2 RdRp all possess the wildtype V557, and static model constructed by manually mutating V557 to Leucine may not appropriately estimate the conformation of V557L upon mutation. In this regard, MD simulations would help to fully sample the conformational space of V557L and explore the orientations of its side chain under dynamic protein environment, which lay the foundation for the investigation of molecular mechanisms.

Again, we would like to thank the reviewer for this insightful comment. In this revision, we have inserted the above discussions and edited the introduction accordingly in the revised maintext. We hope these modifications can clearly illustrate the importance of understanding the "template-dependent" inhibition and the potential role of MD simulations in elucidating the molecular mechanism.

2. The model building process needs to be expanded. This can continue to be in the supplementary methods, but there are major modifications happening to the cryo-EM structures in order for the simulations to be at the desired starting point, the precatalytic state.

2.(1) For example, the 7BZF structure has remdesivir incorporated into primer strand and translocated to the -1 position. Why was this structure used instead of 7BV2 which has remdesivir incorporated but not translocated?

Reply: We thank the reviewer for this good suggestion. In order to exam if the NTP incorporation is directly perturbed at the active site when Remdesivir is present at *+1* site of the template strand, we need to construct a model of pre-catalytic state with NTP binding at the closed active site (Fig. R8A). We chose 7BZF cryo-EM structure as the structural basis because the RdRp is in the post-translocation state with an unoccupied active site (*+1* site), i.e. the 3'-terminal of the nascent strand is at *-1* site (Fig. R8B). This structure provides similar dsRNA conformations (shown in red and cyan in Fig. R8) as in the pre-catalytic state (Fig. R8A), which allows us to directly obtain the NTP and Mg^{2+} ions by structural alignment to the norovirus RdRp (PDBID: 3H5Y) in the pre-catalytic state. The Remdesivir incorporated at *-1* site of nascent strand in 7BZF was modified to ensure the nascent strand contains natural nucleotides. The base pair at the active site (*+1* site) was modified to UTP:A to allow for the subsequent substitution of A with Remdesivir to investigate the inhibitory effect.

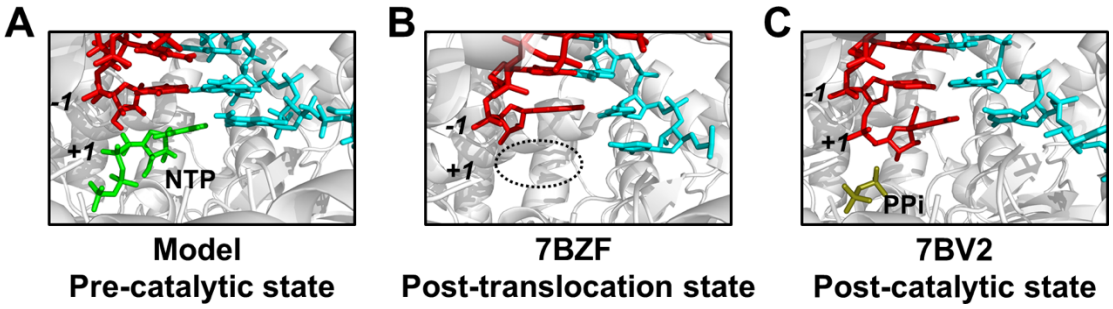

Fig. R8 The dsRNA conformation at the active site in our model and two cryo-EM structures of SARS-CoV-2 RdRp. (A) Our model with NTP (in green) bound at the active site (*+1* site) and not covalently bonded with the 3'-terminal of nascent strand at *-1* site (in red). (B) The 7BZF structure with an unoccupied active site and the 3'-terminal nucleotide of the nascent strand is at *-1* site. (C) The 7BV2 structure with the 3'-terminal of nascent strand (in red) occupying the active site (*+1* site) and the pyrophosphate ion (in dark yellow) is also located at the active site. In (A)-(C), the nascent strand and the template strand are shown in red and cyan, respectively.

The 7BV2 structure demonstrates RdRp in the post-catalytic state with the byproduct pyrophosphate ion (PPi) bound at the active site (Fig. R8C). In this structure, the 3'-terminal of the nascent strand occupies the active site (*+1* site). However, the target model of the pre-catalytic state requires that the 3'-terminal of nascent strand locates at *-1* site and the NTP is at the *+1* site while not covalently bound with the nascent strand (Fig. R8A). Therefore, the dsRNA in 7BV2 structure adopts the conformation different from that in the pre-catalytic state (shown in red and cyan in Fig. R8A and R8C). Although 7BV2 structure could also serve as an alternative basis for model construction

upon multiple-step modifications, the different dsRNA conformational states in 7BV2 structure (Fig. R8C) and in the target model (Fig. R8A) render it more challenging to use 7BV2 as the basis to construct our initial model for MD simulations.

In the revised SI, we have followed the reviewer's suggestion and expand the SI Section 1.1.1 for model building process to justify the choice of the cryo-EM structure as well as the subsequent modifications.

2. (2) How did the structure change after the NTP and Mg cations were added from the Norovirus RdRp and the structure minimized? Is your approach sensitive to differences in starting positions?

Reply: We would like to thank the reviewer for this great comment. As suggested by the reviewer, we have compared the conformations of RdRp complex before and after energy minimization (shown in steelblue and green, respectively, in Fig. R9). No obvious conformational change has been observed with a RMSD as low as ~ 0.6Å when all the C_α atoms, the heavy atoms of nucleotides, NTP and the two Mg^{2+} ions were included for the RMSD calculation.

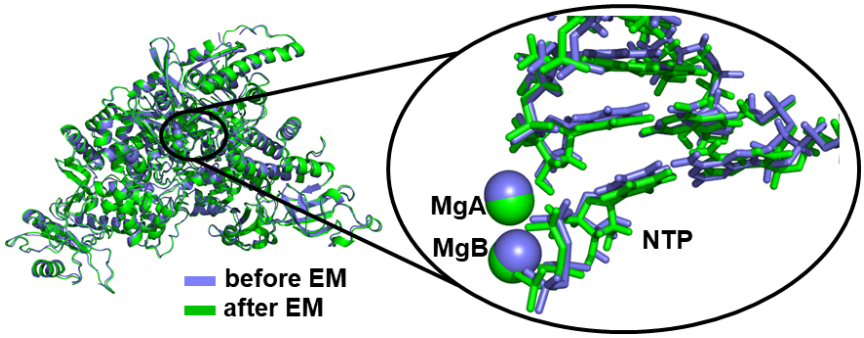

Fig. R9 Comparison of the conformation before (in steelblue) and after (in green) energy minimization.

To include the above comparison, we have inserted Fig. R9 as Fig. S11 and added the following sentence in the revised SI:

"*… No obvious conformational change has been observed with a RMSD as low as ~ 0.6Å (Fig. S11) when all the C_α atoms, the heavy atoms of nucleotides, NTP and the two Mg^{2+} ions were included for the RMSD calculation. …*"

To further examine if our approach is sensitive to the initial positions of NTP and Mg^{2+} ions relative to the dsRNA, we have constructed two new models based on different experimental structures (see Section 4.1 of the revised SI for the details of model construction) and re-examined the stability of the active site by MD simulations. In our current model, the initial positions of NTP and Mg^{2+} ions in the active site were obtained by aligning the 7BZF structure of SARS-CoV-2 RdRp to the norovirus RdRp in the pre-catalytic state (PDBID: 3H5Y) based on the C_α atoms using Pymol programme, as the active site configuration is conserved across the viral RdRps. This model is named as "7BZF-3H5Y". To achieve a new model, we have followed the previous work (*J. Biol. Chem.* 295, 16156-16165, (2020)) to construct the model (named as "6YYT-4WTD") based on another cryo-EM structure of SARS-CoV-2 RdRp in the post-translocation state (PDBID: 6YYT), and used the HCV RdRp (PDBID:

4WTD) in the pre-catalytic state as the reference for structural alignment to obtain the initial positions of NTP and Mg ions. Besides, we have also built an alternative model (named as "7BZF-4WTD") by using the same SARS-CoV-2 RdRp (PDBID: 7BZF) as used in our study but obtaining the NTP and Mg^{2+} ions from HCV RdRp (PDBID: 4WTD). In the two new models, the positions of NTP and Mg ions are a bit different from that in our current model (Fig. R10), which can thus well serve the purpose to examine if our approaches and results are sensitive to the initial model.

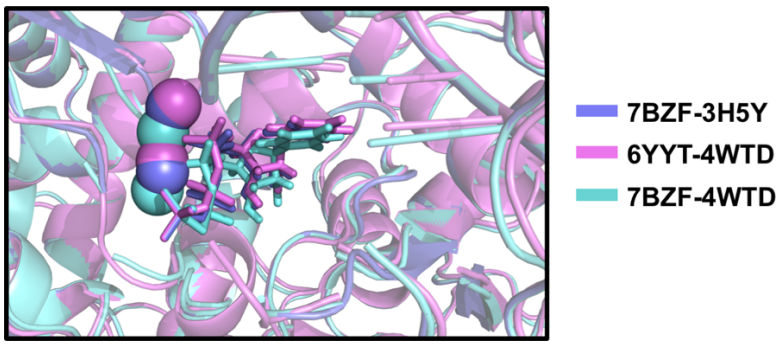

Fig. R10 Comparison of the initial positions of NTP (shown in sticks) and Mg^{2+} ions (shown in spheres) in our model with that in the two new models.

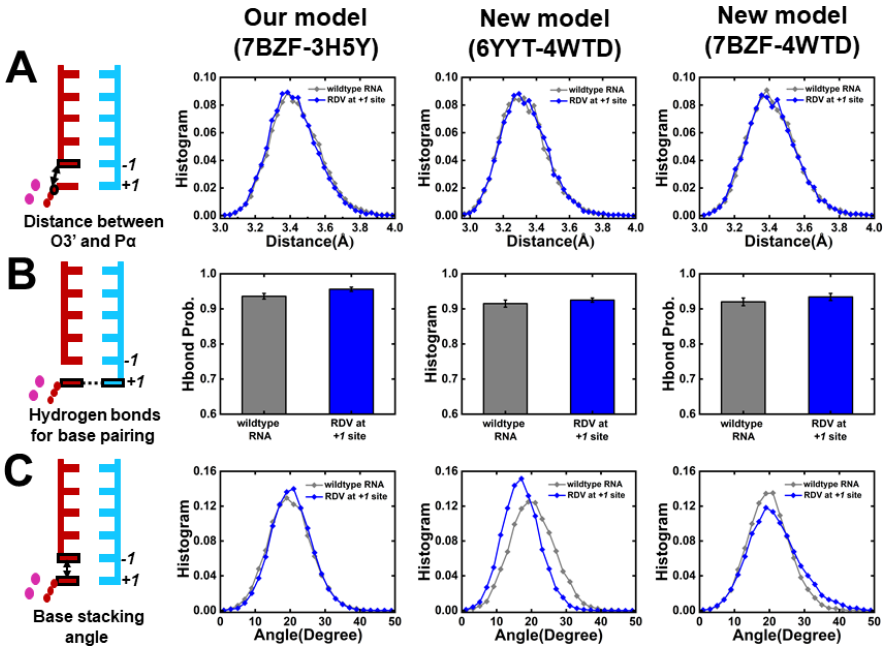

Fig. R11 Examination over the stability of active site using MD simulations initiated from different models. (A) The distance between P_α atom of NTP and the O3' atom of the 3'-terminal nucleotide of the nascent strand. (B) The hydrogen bonding probability for the base pair at the active site. (C) The base stacking angle between NTP and the 3'-terminal nucleotide. In (A)-(C), the first column is the cartoon showing the structural feature under investigation, and the second to fourth columns demonstrate the results for our current model, the "6YYT-4WTD" model and the "7BZF-4WTD" model.

In this revision, we have first performed 5×50ns MD simulations for the two new models ("6YYT-4WTD" and "7BZF-4WTD") and compared the results with that obtained from our "7BZF-3H5Y" model. We have found out that the NTP maintains

the catalytically active conformation in all models (grey lines in Fig. R11A). Besides, the stability of its base pairing and base stacking simulated using the new models also resemble that using the "7BZF-3H5Y" model (shown in grey in Fig. R11B and R11C). Moreover, for each of the new models, we have also embedded Remdesivir at *+1* site of the template strand and performed 5×50ns MD simulations. The results are consistent with the observations based on our "7BZF-3H5Y" model and have further consolidated that the presence of Remdesivir at *+1* site of the template strand seldom directly perturbs the NTP's capability in maintaining the catalytically active conformation and the UTP:Remdesivir base pair is well formed at the active site (shown in blue in Fig. R11).

Overall, although the initial positions of NTP and Mg^{2+} ions in the two new models "6YYT-4WTD" and "7BZF-4WTD" are different from that in our "7BZF-3H5Y" model, MD simulations based on all three models have demonstrated similar NTP stability at the active site, indicating that the active site configuration is seldom influenced by the initial positions of NTP and Mg^{2+} ions.

We would like to thank the reviewer again for this useful comment. In the revision, we have added Fig. R10 as Fig. S12 in the revised SI and inserted a new SI Section 4 to elaborate the details of model construction and MD simulations for the two new models. The results of the two new models in Fig. R11 have been inserted to the revised SI as Fig. S5A-C. Beside, we have included the above discussions in the revised maintext as follows:

In maintext, "…*We also noted that the previous study has used an alternative cryo-EM structure of SARS-CoV-2 RdRp (PDBID: 6YYT) as the basis to construct the model. To further examine if the discrepancy of the base pairing stability is originated from the modelling using different experimental structures, we have constructed two extra models by referring to the previous study. MD simulations have been performed to examine the stability of the active site as well as the position of A558 relative to the template strand nucleotide (Fig. S5, see SI Section 4 for details). We have found out that even though different experimental structures were used for the initial modelling, the UTP: Remdesivir base pair at the active site is always well maintained, and A558 would move away from Remdesivir, further consolidating the robustness of our results. ...*"

3. The discussion would greatly benefit from better integration with the published experimental and modeling results.

3.(1) For example, modeling done by Tchesnokov et al. 2020 suggested that remdesivir is a poor template for the incoming UTP because base pairing is compromised. Yet your simulation predicted that base pairing is stable. Does the protein backbone require large shifts to prevent clashing with A558?

Reply: We would like to thank the reviewer for this insightful suggestion. The model by Tchesnokov et al. 2020 was constructed based on another SARS-CoV-2 structure (PDBID: 6YYT) and the positions of NTP and Mg^{2+} ions were obtained by structural alignment to HCV RdRp structure (PDBID: 4WTD). 6YYT structure is an alternative

SARS-CoV-2 RdRp structure in the post-translocation state, the same state as that of the 7BZF structure used in our model. The 4WTD structure is HCV RdRp in the pre-catalytic state, the same state as that of the norovirus RdRp (PDBID: 3H5Y) used in our modelling. Therefore, although the structural basis used by Tchesnokov et al. 2020 to construct the model are different from that used in our model, the experimental structures adopt the same functional states in the nucleotide addition cycle, which assumes similar active site configurations.

However, as pointed out by the reviewer, the model constructed by Tchesnokov et al. has suggested that the UTP:Remdesivir base pair is compromised while our simulations have demonstrated a stable base pairing. To further examine if the different initial models would lead to different active site stability, we have followed the previous work to use the SARS-CoV-2 structure (PDBID: 6YYT) and HCV RdRp structure (PDBID: 4WTD) as the basis to construct the model (named as "6YYT-4WTD", the same model as used in the response to comment #2(2) of reviewer #2) with natural adenosine or Remdesivir at *+1* site of the template strand. After energy minimization, we have observed that the UTP:Remdesivir base pair at the active site can be well formed, similar to the scenario for the UTP:A pair (Fig. R12). This observation is different from that shown in the previous work even though we have used the same experimental structures as the previous work. We anticipate such a discrepancy could be attributed to the different energy minimization algorithms/set-up. In specific, we used the steepest descent algorithm to perform the energy minimization in Gromacs program while Tchesnokov et al. achieved the optimization via minimization and conformational searches using the Schrödinger suite of programs.

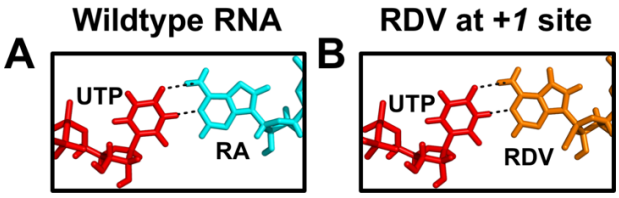

Fig. R12 The energy minimized (EM) conformation of the "6YYT-4WTD" when adenosine (RA)/Remdesivir is present at *+1* site of the template strand. (A) UTP is base paired with RA in the EM conformation of the "wildtype RNA" system. (B) The UTP:Remdesivir base pair is formed in the EM conformation of the "RDV at *+1* site" system.

Although the energy minimized conformation may vary due to the different algorithms, MD simulations could help to fully relax the system and estimate the thermal stability of base pair under dynamic protein environment, which is closer to the physiological condition. In this regard, MD simulations have also been performed for "6YYT-4WTD" model and the UTP:Remdesivir base pair is still well maintained (Fig. R11B, see response to comment #2(2) of reviewer #2 for details). Moreover, we have observed that the backbone of A558 has largely shifted away from Remdesivir to avoid the steric clash (Fig. R13). In specific, the distance between A558 and Remdesivir is 6.05Å±0.3Å, obviously larger than that between A558 and adenosine (3.5Å±0.16Å). This observation is consistent with the previous finding by Tchesnokov et al.

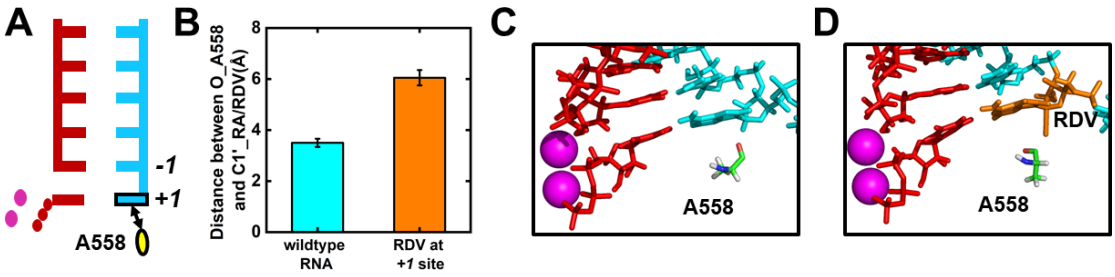

Fig. R13 A558 is moving away from Remdesivir as shown by MD simulations initiated from "6YYT-4WTD" model. (A) Cartoon showing the distance was computed between A558 and the RA/RDV at the *+1* site of the template strand. (B) The distance between the backbone oxygen atom of A558 and the C1' atom of RA in the "wildtype RNA" system (in cyan) is compared with that between A558 and Remdesivir in the "RDV at *+1* site" system (in orange). (C)-(D) Representative MD conformation showing the position of A558 relative to RA in (C) and RDV in (D).

Overall, in this revision, we have used the same experimental structures as previous work for the modelling. However, we have observed that the base pair between the UTP and Remdesivir could still be formed not only in the energy minimized conformation, but also in the MD conformational ensemble. Besides, our simulations have demonstrated that the backbone of A558 requires an obvious shift to avoid the steric clash with Remdesivir and this observation is consistent with that shown by the previous work of Tchesnokov et al..

In this revision, Fig. R13A and R13B have been included in Fig. S5D of the revised SI. Besides, the above discussions have been included in the inserted sentences of the revised maintext as shown in the response to the comment #3(1) of reviewer #2.

3. (2) Is there experimental evidence for the prediction that inhibition is due to +2 to +1 translocation versus Tchesnokov's proposal of compromised base-pairing?

Reply: We are sorry that so far there has been no direct experimental evidence for our prediction that the inhibition is due to the hindered translocation from *+2* to *+1* site rather than the compromised base pairing. Even so, our simulations have shown that the translocation obstacle can be bypassed when V557 is mutated to Leucine and the inhibitory effect acted by Remdesivir is thus reduced (Fig. 3 and Fig. 5 in the original and revised maintext). This finding is consistent with the experimental evidence that RdRp upon V557L mutation would become drug resistant to Remdesivir. Moreover, we have also explored if V557L mutation would influence the base pairing stability at the active site (Fig. S4 in the original SI and Fig. S8 in the revised SI), and found out that the stability of UTP:Remdesivir base pair resembles that in the wildtype RdRp, suggesting V557L mutation seldom exerts extra influence on the base pairing stability.

Overall, although there is no direct experimental evidence, our prediction that inhibition is due to *+2* to *+1* translocation can well distinguish the effect of V557 versus V557L on NTP incorporation and match with the experimental data, which thus could offer a promising mechanism for "template-dependent" inhibition.

We would like to thank the reviewer for raising this question and in the revision, we have inserted the following sentence in the revised maintext:

"*… Although there is no direct experimental evidence to support that the inhibition is due to the translocation rather than the compromised base pairing, our prediction can well distinguish the effect of V557 versus V557L on NTP incorporation as observed in the biochemical experiment, which thus could offer promising mechanism for "template-dependent" inhibition. …*"

3. (3) I understand the translocation pathway saw a difference between wild-type RdRp and V557L, but how do we know the conformation in Fig 3G is not just an artifact of a high-energy contact as remdesivir is translocated.

Reply: We thank the reviewer for this good comment. Although the preliminary translocation pathway was generated by inputting external energies, the conformation in Fig. 3G has been relaxed by straightforward MD simulations and the unfavorable contact would have been removed. To further validate if the conformation in Fig. 3G is stable rather than as a high-energy and low populated conformation, we have collected the MD conformations initiated from the translocation intermediates with Remdesivir from *+2* to *+1* site and performed K-center clustering based on the conformational similarity. In specific, the heavy atoms of V557, Remdesivir and its upstream nucleotide were included in the measurement of conformational similarity. The results of clustering have demonstrated one cluster with dominant population (67.6%) while each of the remaining two clusters possesses ~15% of MD conformations (Fig. R14). Interestingly, the highest populated cluster shows the largest base stacking angle (Fig. R14A), i.e., the base of Remdesivir is significantly tilted relative to the base of the upstream nucleotide with the mean angle of 65.2°±16.1°. The other two clusters are showing the mean angles of 56.5°±13.1° and 14.7°±10.4°, respectively (Fig. R14B and R14C). This analysis has suggested that the conformation as shown in Fig. 3G with an obviously tilted base relative to its upstream nucleotide is a highly populated and representative conformation rather than a high-energy conformation along the translocation pathway for wildtype RdRp with V557.

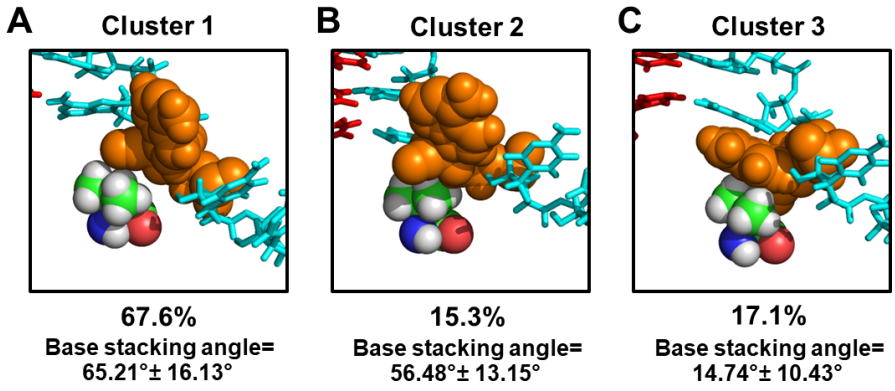

Fig. R14 K-center clustering based on the conformational similarity of the MD conformations initiated from the translocation pathway of Remdesivir from *+2* to *+1* site in wildtype RdRp. The representative conformation from each cluster is shown. The representative conformation is defined as the conformation with the base stacking angle close to the mean value of the cluster. Below each representative conformation, the population of the respective cluster is labelled. The mean values and

standard deviations of the base stacking angles for the conformations within each cluster were also computed and displayed.

In this revision, we have inserted Fig. R14 as Fig. S14 and included the above discussions as SI Section 3.4. Besides, the following sentence has been inserted into the revised maintext:

"*… Further calculations have validated that the conformation as shown in Fig. 3G with an obviously tilted base is a highly populated and representative conformation along the translocation pathway in SARS-CoV-2 RdRp with V557, as K-center clustering based on the conformational similarity has shown that ~83% of MD conformations fall into the clusters with the mean angle > 50° (see SI Section 3.4 for details). …*"

4. The manuscript would benefit from copyediting, ie. "health crisis" vs. "healthy crisis."

Reply: We thank the reviewer for pointing this out. In this revision, we have checked and copyedited the manuscript thoroughly.

5. The figures could be greatly enhanced with better labeling and 3D structures. For example, figure 2 could use structures to illustrate the measurements like in figure 4.

Reply: We thank the reviewer for this great suggestion and accordingly, we have added the 3D structures and the labelling for the *+1* and *-1* sites in Fig. 2 of the revised maintext.