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Inhibition Mechanism of Remdesivir on Reproduction of SARS-CoV-2 and *Ebola* Viruses

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Abstract

Remdesivir is an antiviral drug initially designed against the *Ebola* virus. The results obtained with it both in biochemical studies *in vitro* and in cell line assays *in vivo* were very promising, but it proved ineffective in clinical trials. Remdesivir exhibited far better efficacy when repurposed against SARS-CoV-2. The chemistry that accounts for this difference is the subject of this study. Here, we examine the hypothesis that remdesivir monophosphate (RMP)-containing RNA functions as a template at the polymerase site for the second run of RNA synthesis, and as mRNA at the decoding center for protein synthesis. Our hypothesis is supported by the observation that RMP can be incorporated into RNA by the RNA-dependent RNA polymerases (RdRp) of both viruses, although some of the incorporated RMPs are subsequently removed by exoribonucleases. Furthermore, our hypothesis is consistent with the fact that RdRp of SARS-CoV-2 selects RMP for incorporation over AMP by 3-fold *in vitro*, and that RMP-added RNA can be rapidly extended, even though primer extension is often paused when the added RMP is translocated at the i+3 position (with i the nascent base pair at an initial insertion site of RMP), or when the subsequent nucleoside triphosphates (NTPs) are below their physiological concentrations. These observations have led to the hypothesis that remdesivir might be a delayed chain terminator. However, that hypothesis is challenged under physiological concentrations of NTPs by the observation that about three quarters of RNA products efficiently overrun the pause.

Graphical Abstract

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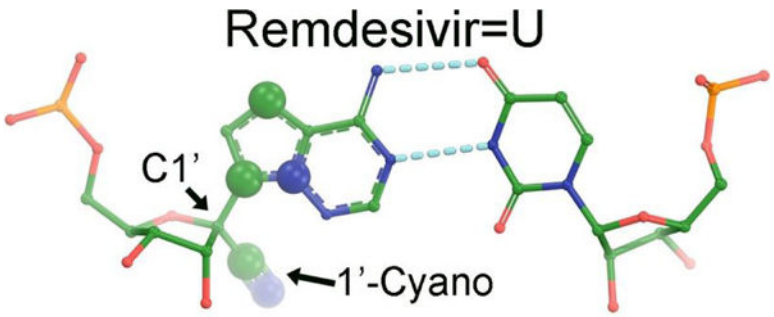
Author Contributions

J.W. designed and executed the experiments and wrote the draft manuscript. All authors were involved in the analysis and interpretation of results and writing of the manuscript.

Competing interest statement

The authors declare that there is no competing interest in this study



Topics of Content (TOC). Chemical structure and base pairing of remdesivir

Keywords

Remdesivir; chain terminator; delayed chain termination; poly(A) tail; RMP-containing template; RMP-containing primer; translating ribosome; decoding center; codon-anticodon base pairing; Ebola virus; mouse hepatitis virus

Introduction

Remdesivir (R) is an analogue of adenosine (A), capable of forming Watson-Crick base pairs with uridine (U).[1, 2] It has a cyano substitution at the H position of the C1' center, a strong electron-withdrawing group that destabilizes the glycosidic C1'-N9 bond. To prevent hydrolysis of the glycosidic bond, C and N atoms within its aromatic base ring are repositioned for it to become 4-aza-7,9-dideazaadenosine such that a C-C bond occupies the equivalent glycosidic bond. This repositioning does not alter the hydrogen bonding capability of its base so it effectively mimics adenosine by hydrogen bonding to uridine mono-phosphate (UMP). Within the context provided by the active site of the polymerase (pol) within the pol replication complex, there is very little electronic difference of the base between remdesivir monophosphate (RMP) and adenosine monophosphate (AMP).

Remdesivir exhibited promising results both *in vitro* and *in vivo* studies as well as in animal models for treatment of *Ebola* virus; however, it exhibited very little efficacy in clinical trials.[3, 4] Remarkably, it exhibited much better efficacy when repurposed for treatment of patients infected with SARS-CoV-2 although the mechanism of the inhibition remains uncertain.[5, 6] Therefore, understanding how remdesivir inhibits the RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2 remains an outstanding challenge that could provide valuable insights to development of new drugs with even better efficacy.

Base selectivity for RMP over AMP by RNA polymerases

Remdesivir exhibits very low toxicity in practice, as human RNA polymerases select AMP over RMP for nucleotide incorporation by as much as three orders of magnitude.[4] RdRp from both SARS-CoV-2 and *Ebola* virus are able to efficiently incorporate RMP into RNAs, making it a potential inhibitor of viral multiplication.[3, 7–9] In fact, RdRp of SARS-CoV-2 selects RMP for nucleotide incorporation over AMP by about 3-fold *in vitro* while RdRp of

Ebola selects AMP over RMP, also by 3-fold.[8] Thus, there is a 9-fold difference in RMP selectivity of the two pols.

An initial working hypothesis has been that remdesivir is a direct-acting nucleotide analog chain terminator that targets specifically viral RdRp. That proposal has been based on the observation that remdesivir can be selectively incorporated into viral RNAs and escaped from excision by exoribonuclease to some extent, but not into host RNAs. However, remdesivir has an extendable 3'-OH so that it differs from the classic chain terminators such as 3'-deoxy version of analogs or AZT that lack an extendable 3'-OH. Here, we propose that the 3'-deoxy, 3'-F, 3'-NH_2, or 3'-N_3 versions of remdesivir could be developed to be more effective inhibitors, assuming that they can be efficiently converted into the triphosphate form by the cellular machinery. These new true chain terminators could completely block, or significantly slow down, the synthesis of viral RNAs but should not affect the synthesis of host RNAs.

The chain termination hypothesis for remdesivir was supported by initial cell-based assays. An analysis of the level of the total RNAs of the SARS-CoV-2 infected cells showed that overall, 80% of the total RNAs were virally encoded RNA in the absence of treatment of remdesivir, whereas the percentage was reduced to ~ 10% when the cells were treated with remdesivir,[10, 11] suggesting that remdesivir treatment significantly reduced the level of viral RNAs. In cell line assays *in vivo*, the amount of remdesivir needed to reduce 50% of viral RNA levels increased when the exoribonuclease-deficient SARS-CoV-1 or *Ebola* virus were studied, suggesting that RMP may act as a chain terminator and that the exoribonuclease was able to remove some of added RMP chain terminators so that it can restart primer extension. However, the level of viral RNA does not always correlate well with the level of viral replication and viral infection in the presence of antiviral drugs,[12] because the level of viral infection is proportional to the number of cells being infected and because it is unclear whether all viral RNAs are functional for viral multiplication. Alternatively, viral RNAs synthesized in the presence of remdesivir may be unstable and degraded rapidly, which would be contradictory to the chain termination hypothesis.

A hypothesis of delayed chain termination

In vitro observations show that purified nsp12 or RdRp from SARS-CoV-2 incorporates RMP by ~3-fold more efficiently than AMP, and that the 3'-hydroxyl of added RMP can also serve as a new substrate for addition of the next Watson-Crick basepaired nucleotide,[8, 9] ruling out the original hypothesis that remdesivir serves as a chain terminator. It was observed that primer extension for the next three nucleotides was very efficient even after the concentrations of the next NTPs were artificially decreased.[8, 9, 13, 14] However, it has been observed that primer extension for the fourth nucleotide was stopped leading to a revised hypothesis, called a delayed chain termination.

Intracellular ATP concentration is the highest among four NTPs and varies from 3 to 10 mM, with the cellular concentrations of three other NTPs of about 0.5 mM.[15–17] Therefore, the question was raised as to whether the primer extension remained stalled under the physiological concentrations of NTPs. A careful quantification showed that about 3/4 of

RNA products rapidly extended primers as if RMP were never incorporated or as if RMP behaved exactly the same as AMP, and that only 1/4 of RNA products failed to be fully extended.[13] If exoribonuclease were added, the stalled RNA products would likely have been hydrolyzed so that they could be further extended after removing RMP. Therefore, the paused primer extension observed *in vitro* at the *i*+3 site after RMP addition (with *i* the nascent base pair at an initial insertion site of RMP) is short lived and not likely to be biologically relevant to the reduced level of viral RNAs observed in the infected cells.

RNA synthesis during transcription-replication in SARS-CoV-2 and Ebola virus

SARS-CoV-2 is a positive-sense single-stranded (ss) RNA virus.[18, 19] The ssRNA genome of SARS-CoV-2 is highly structured with a vast network of secondary structures, duplex stems, hairpins, and pseudoknots, stabilized by a large number of bound nucleocapsid proteins.[20–22] The genome is properly positioned within the pre-packaged functional replication-transcription complex (RTC) inside the virus. After the virus enters permissive host cells where a pool of NTPs is available, RdRp rapidly transcribes a negative-strand fusion RNA product in a discontinuous manner, known as a transcription-intermediate (TI). Each TI serves as a template for repetitive synthesis of tens to hundreds copies of different positive-sense viral mRNAs for synthesis of viral proteins. All sub-genomic (sg) transcripts have identical 5'- and 3'-sequences whereas they differ in various deletions of middle sequences, flanked by a pair of transcription-regulatory sequences (TRS), through a strand-switching mechanism or jumping events. After accumulations of all necessary viral proteins, RdRp transcribes another specialized negative-strand replication-intermediate (RI) as a template for faithful synthesis of new genomic RNA in its entirety for viral packaging without any deletion. In the virally infected cells, the majority of viral RNAs are positive-sense mRNAs. The amount of negative-sense RNAs is very low (only a few percent) and difficult to study.[18] Yet, they are essential as templates for making viral mRNAs and new genomic RNAs. Different from viral mRNAs which are often short-lived and rapidly turned over, both TI and RI are long lived, resistant to RNA degradation, likely due to some specific protections by the RTC to both of their 5'and 3' ends. An unaddressed important question is whether RMP-containing RNA templates can be copied. If not, it may explain why the level of viral rRNAs is reduced in the infected cells after they were treated with remdesivir. The presence of RMP in the template could also result in high noise of random transcriptional jumping events or uncontrolled strand-switching in positive-sense RNAs and disrupt the normal functions of viral mRNAs.[11]

Ebola is a negative-sense ssRNA virus.[23] The synthesis of its viral mRNAs does not involve a transcription-intermediate as a template for synthesis of viral mRNAs as in the case of SARS-CoV-2 because viral mRNAs are directly transcribed from the RNA genome. Therefore, the mechanism of transcription-replication cycle differs between SARS-CoV-2 and *Ebola* virus, as does the relative importance of the first and second runs of RNA synthesis. If remdesivir directly acts on the viral intermediate RNAs as templates, its effects on inhibition of viral multiplication will differ against these two viruses. In addition, the genome of SARS-CoV-2 is A and U enriched with A, U, G, and C contents of 30%,

32%, 20%, and 18%, respectively, whereas that of *Ebola* virus is enriched in G and C contents.[24–26] Given the fact that RdRp of SARS-CoV-2 prefers RMP over AMP whereas RdRp of *Ebola* virus prefers AMP over RMP, these differences could be amplified to result in different efficacies of remdesivir for treatment of patients infected with one of these two viruses.

Structures of the replication complexes of RNA polymerases with RMP-containing primer/template RNA duplexes

The first structure of the replication complex of SARS-CoV-2 RNA polymerase with RMP-containing primer/template (P/T) was obtained upon incubation with remdesivir triphosphate (RTP) opposite to poly(U) template.[27] It was found that the pol was able to add only single RMP with the pyrophosphate product remaining bound (Fig. 1a). The added RMP did not appear to be extended by a second RMP, a situation that differs from results of biochemical studies using a non-poly(U) template where primer extension after RMP is very efficient. An implication of this observation is that the genomically encoded poly(A) tails of viral mRNAs cannot be fully synthesized and are often shortened in the presence of remdesivir. This is because the synthesis of genomically encoded 33-nucleotide poly(A) tails of viral mRNAs is carried out by RdRp using 5'-poly(U) negative-sense (PUN) template of transcription intermediate (TI),[28] whereas these of cellular RNAs are synthesized by cellular poly(A)-polymerase. Without the protection of poly(A) tails, viral RNAs will be degraded very rapidly, which would also explain the reduced level of viral RNAs in the SARS-CoV-2 infected cells after being treated with remdesivir. In fact, many viruses evolve an elaborate mechanism for protection of the poly(A) tails of their viral RNAs.[29–32]

Many additional structures of replication complexes with RMP added at different positions of the primer strands were obtained using chemically synthesized RMP-containing primers or enzymatically synthesized by the pols (Fig. 1).[13, 14, 33–35] Similarly, primer extension assays with RMP added at different positions of the primer strands were studied using both chemically synthesized and enzymatically synthesized primers. Results are nearly identical regardless of the sources of RMP-containing primers. As expected, one-nucleotide extended RMP-containing primer forms a Watson-Crick base pair to the template as does exactly the AMP-containing primer (Fig. 1b).[27] At this position, the extra cyano substitution is not in direct contact with any sidechain of the pol, which is why RMP-added primer can be rapidly translocated from the insertion "*i*" site to the *i*+1 site and be extended efficiently. Nevertheless, the substituted cyano group may interact with the pol through ordered water molecules, which remain unresolved due to relatively low resolution of cryo-electron microscopic (cryo-EM) structures. After incorporation of NMP and release of pyrophosphate, the synthesized RNA product is translocated to the *i*+1 position, which is known as the post-translocated product state with a vacant NTP binding site ready for accepting the next incoming NTP (Fig. 1c).[27]

RdRp selects incoming NTPs according to Watson-Crick base pairing principles opposite to the templating nucleotide as does DNA pols although the base selectivity for nucleotide incorporation by RNA pols is often much poorer than the base selectivity by replicative

DNA pols. It is well known that many conformational changes of DNA pols occur upon initial binding of the Watson-Crick base paired incoming dNTP, formation of the closed replication complex, alignment of substrates, chemical step of nucleotide addition, and release of pyrophosphate, followed by the translocation of the P/T duplex product for the next nucleotide addition.[36] When a non-Watson-Crick base paired dNTP binds, each of these events becomes slower so that the incorrect dNTP will be preferably rejected. Although it is likely that many equivalent conformational changes may also occur in RNA pols but with smaller amplitudes, they have not yet been fully characterized. After misincorporation of a non-Watson-Crick base paired NMP, primer extension is often stalled so that it can be removed either by pyrophosphorylysis or by exoribonuclease. When it is removed by exoribonuclease, mispaired NMP must first become unpaired and be transferred into the exoribonuclease active site for hydrolysis. If RMP remains Watson-Crick base paired and if RMP-added primers can be rapidly extended with the next Watson-Crick base paired NMPs, both of which are true for RdRp of SARS-CoV-1 and SARS-CoV-2, it is inevitable that the synthesized viral RNAs will contain a high level of RMPs.

A comparison of the i+1 base pair between two pol replication structures with RMP-containing primer at two different positions, one at the i position and the other at the i+1 position, shows that the RMP/UMP base pair is noticeably displaced away from the pol, relative to a normal Watson Crick base pair (> 0.5 Å) (Fig. 1d).[27] As a result, the nascent base pair next to the i+1 positioned RMP-containing P/T base pair is also displaced from the pol (Fig. 1e).[27] This conformational state likely differs from the catalytically active state. When comparing with the post-translocated state, resulting in a vacant NTP binding site, the i+1 base pair appears to be displaced towards the pol (Fig. 1f),[27] likely making a strong interaction with the pol and thus driving the translocation process forward. After incorporation of RMP, the extra cyano substitution appears to prevent the RMP/UMP base pair at the i+1 position from being displaced towards the pol, as seen in the normal Watson-Crick base pair. This may explain why the translocation process after RMP incorporation is not spontaneous and the NTP-binding site is not yet vacant for accepting the next NTP. Therefore, it would require a higher concentration of the next NTP to drive the translocation forward after RMP incorporation.

The comparison above was based on superposition of the pol subunit between different complexes (Fig. 1).[27] Within experimental errors, so far there is no large open-to-closed conformational change of the Fingers domain observed in these structures in contrast with those in DNA pols. Comparison with other structures of this pol further supports this conclusion.[13, 14, 27, 33, 34] It is likely that some subtle conformational changes will occur for base selection of nucleotide incorporation, which would require much higher resolution to resolve than that observed in the current cryo-EM structures.

Models of RMP-containing RNA as a template strand at the polymerase site

A key feature of remdesivir is the cyano substitution at its C1’ position. This substitution can be easily modeled computationally according to the tetrahedral geometry of the C1’ center and known bond lengths to any position of nucleotides in any of known pol replication complex containing a P/T duplex. A systematic analysis of these structures of the RdRp

from SARS-CoV-2 shows that there are two common features as exemplified in modeling of the 7bv2 structure (Fig. 2).[27] As discussed elsewhere,[8, 14] during the translocation of the RMP between the *i*+3 and *i*+4 site, the side chain of Ser861 of RdRp appears to become a roadblock, which explains a pause during primer extension. The closest interatomic distance between them is about 2.5 Å (Fig. 2e).[27] Translocation could still occur if the P/T RNA duplex is slightly displaced away from Ser861 when there is enough driving energy, as in the presence of high concentration of the next incoming NTP.

When the cyano substitution is modeled onto the nucleotides of the template strand (Fig. 2),[27] it is found that when it is in either the *i* or *i*+1 site there are stereochemical clashes. The interatomic distance between the backbone carbonyl group of Ala588 and the cyano substitution at the *i* site is 1.1 Å, which is a severe stereochemical clash, and it is 2.1 Å between the backbone carbonyl group of Ser682 and the cyano substitution at the *i*+1 site. Given the importance of substrate alignment of Watson-Crick base pairs at the *i* site, the severe clash of 1.1 Å at the templating RMP will cause severe misalignment of substrates. We predict that the RMP-containing template cannot be copied by RdRp of SARS-CoV-2. Therefore, the second run synthesis of viral mRNAs is reduced after incorporation of any RMP into the transient template strand during the first run of RNA synthesis.

Our modeling shows that the cyano substitution at the *i* site of the template strand has a stereochemical clash with the backbone carbonyl of Ala558 within the V557/A558/G559 stretch (Fig. 2).[27] This clash would explain the inhibition of UMP incorporation opposite to RMP at the templating position. Interestingly, the mutation V557L reduces UMP incorporation opposite to AMP by 3-fold but improves it opposite to RMP by 5-fold. This mutation appears to counteract some inhibitory effects of the second run of RNA synthesis opposite the RMP-containing template. *In vitro* selection experiments resulted in special mutations that conferred a reduced susceptibility to remdesivir in two viral RNA polymerases.[10, 37] These are F548S in *Ebola* viral lineage and V557L in the mouse hepatitis virus (MHV), each at the equivalent position of V557 of SARS-CoV-2 viral polymerase.

Models of RMP-containing RNA as a mRNA at the decoding center of protein synthesis

A small fraction of the transient RNA templates during the first run of RNA synthesis may be free of RMP incorporation even in the presence of remdesivir. These templates can be used for synthesis of a large quantity of viral mRNAs. However, it is inevitable that a large fraction of viral mRNA will also contain RMPs when in the presence of remdesivir. We carried out similar modeling of cyano substitutions at each of three codon sites of mRNA at the decoding center of the translating ribosome using the 7k00 structure (Fig. 3).[38] At each codon position, the cyano group appears to overlap with the ribosomal nucleotides. Thus, we predict that translation will be stalled or slowed down when encountering the RMP-containing mRNAs.

Inside the cell, coronaviral mRNAs are loaded onto the ribosome by an apparatus that recognizes and interacts with both the 5’-caps and 3’-poly(A) tails.[39, 40] Once loaded, translating mRNAs are scanned for the translation initiation codon “AUG”, which is

sequestered by the secondary structure and surrounded by a poor Kozak context for coronavirus viral mRNAs,[41] suggesting a possibly inefficient leaky scanning mechanism for translation initiation. This could be a reason why a large number of copies of coronaviral mRNAs are produced in the infected cells for synthesis of viral proteins. Once bound to the ribosome, viral mRNAs are also protected by the ribosome against RNA degradation. Evidence exists, as discussed above, that remdesivir may impair the synthesis of full-length 3'-poly(A) tail of viral mRNAs. Without a 3'-poly(A) tail or with shortened poly(A) tails, viral mRNAs cannot be properly loaded onto the ribosome for translation initiation, and thus they are rapidly degraded. A net consequence of remdesivir's action appears to deplete the pool of NTPs through a futile RNA synthesis-RNA degradation cycle. This novel understanding of remdesivir's action could provide a unique avenue for development of new antiviral drugs as a silver bullet to specifically find the SARS-CoV-2 infected cells, to deplete NTPs in these cells, and to kill them alongside with the virus.

Concluding remarks

Upon analysis of existing structures and examination of recent literature in this study, we have raised an issue with the commonly circulated mechanism that remdesivir is a chain terminator or a delayed chain terminator as a nucleotide analog inhibitor for RNA-dependent RNA polymerase of SARS-CoV-2. Evidence for that hypothesis appears relatively weak. Alternative experimentally testable hypotheses for the mechanism have been put forward, which are based on existing observations that remdesivir may affect the second run of RNA synthesis more than the first run and that it may also impair viral protein synthesis when viral mRNAs contain RMPs. In addition, nothing is known as to whether remdesivir will also inhibit the synthesis of RNA primer by the primase of SARS-CoV-2 or interfere with host tRNA synthetases or other proteins. These new hypotheses are inspired by results of our structural analysis and will stimulate many new experiments.

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ABBREVIATIONS

RdRp	RNA-dependent RNA polymerase
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
RMP	remdesivir mono phosphate
RTP	remdesivir triphosphate
P/T	primer/template

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SYNOPSIS TOC:

Alternative mechanisms other than delayed chain termination seem to better explain existing data on remdesivir on inhibition of reproduction of SARS-CoV-2.

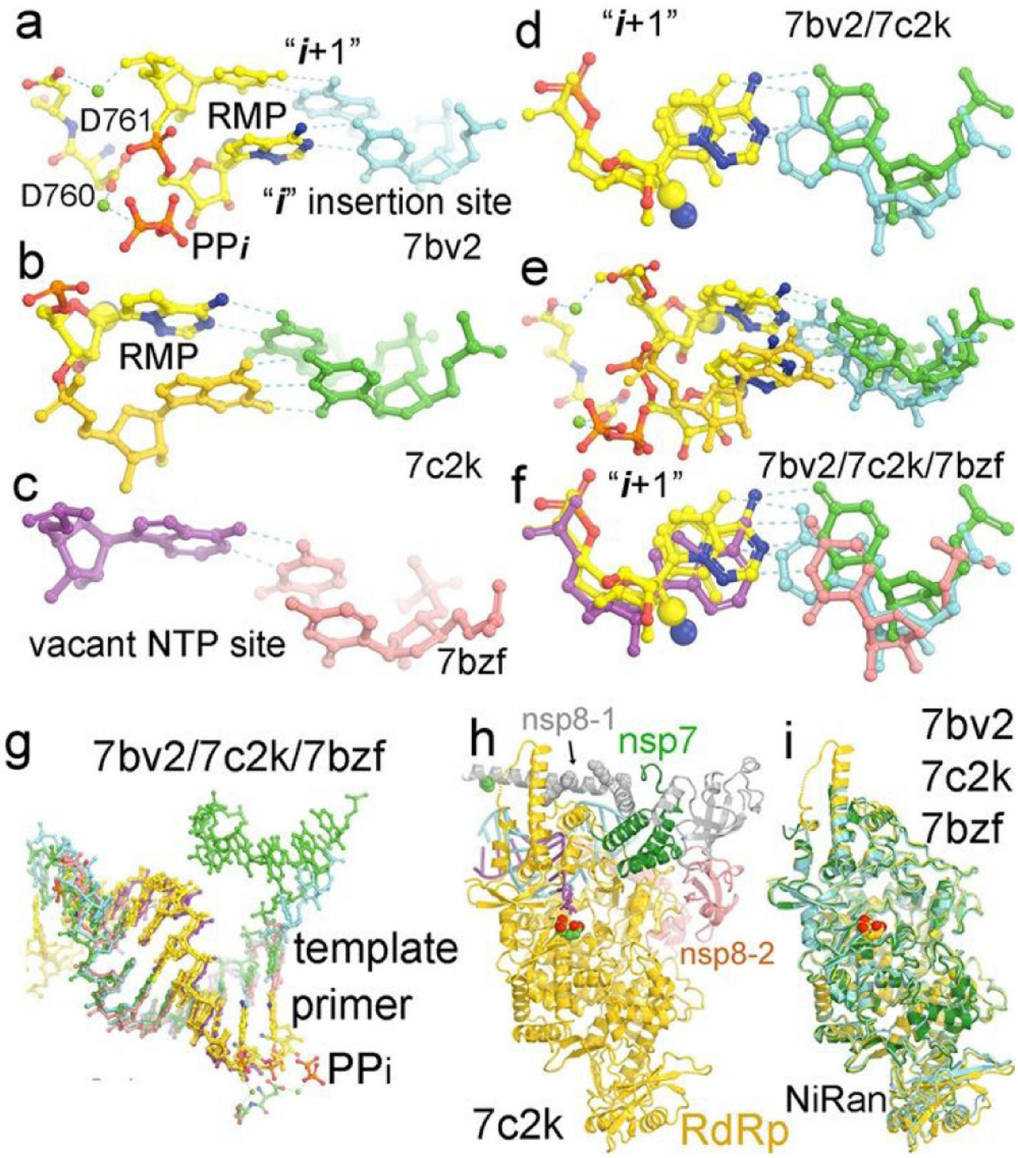

Figure 1.

Conformations of RMP-containing primers in P/T replication complex of RdRp of SARS-CoV-2. (a) The 7bv2 structure with RMP at the "*i*" site and the pyrophosphate (PPi) bound. Two catalytic carboxylates D760 and D761 and two divalent metal ions are shown. (b) The 7c2k structure with RMP at the *i*+1 site in the pre-translocated product complex. (c) The 7bzf structure of a post-translocated product complex with a vacant NTP-binding pocket. (d) Comparison of the 7bv2 and 7c2k structures at the *i*+1 site. (e). Comparison of the 7bv2 and 7c2k structures at both the *i* and *i*+1 sites. (f) Comparison of the 7bv2, 7c2k and 7bzf structures at the *i* site. (g) Comparison of the 7bv2, 7c2k and 7bzf structures for the entire primer/template RNA duplexes. (h) The 7bv2 structure with one nsp8 in grey (nsp8–1) and the second nsp8 (nsp8–2) in salmon, nsp7 in green, and P/T in cyan, and the polymerase in gold and two catalytic carboxylates in large CPK models. (i) The superposition of the 7bv2,

7c2k, and 7bzf polymerase structures, which is a basis of comparison of corresponding P/T RNA duplexes.

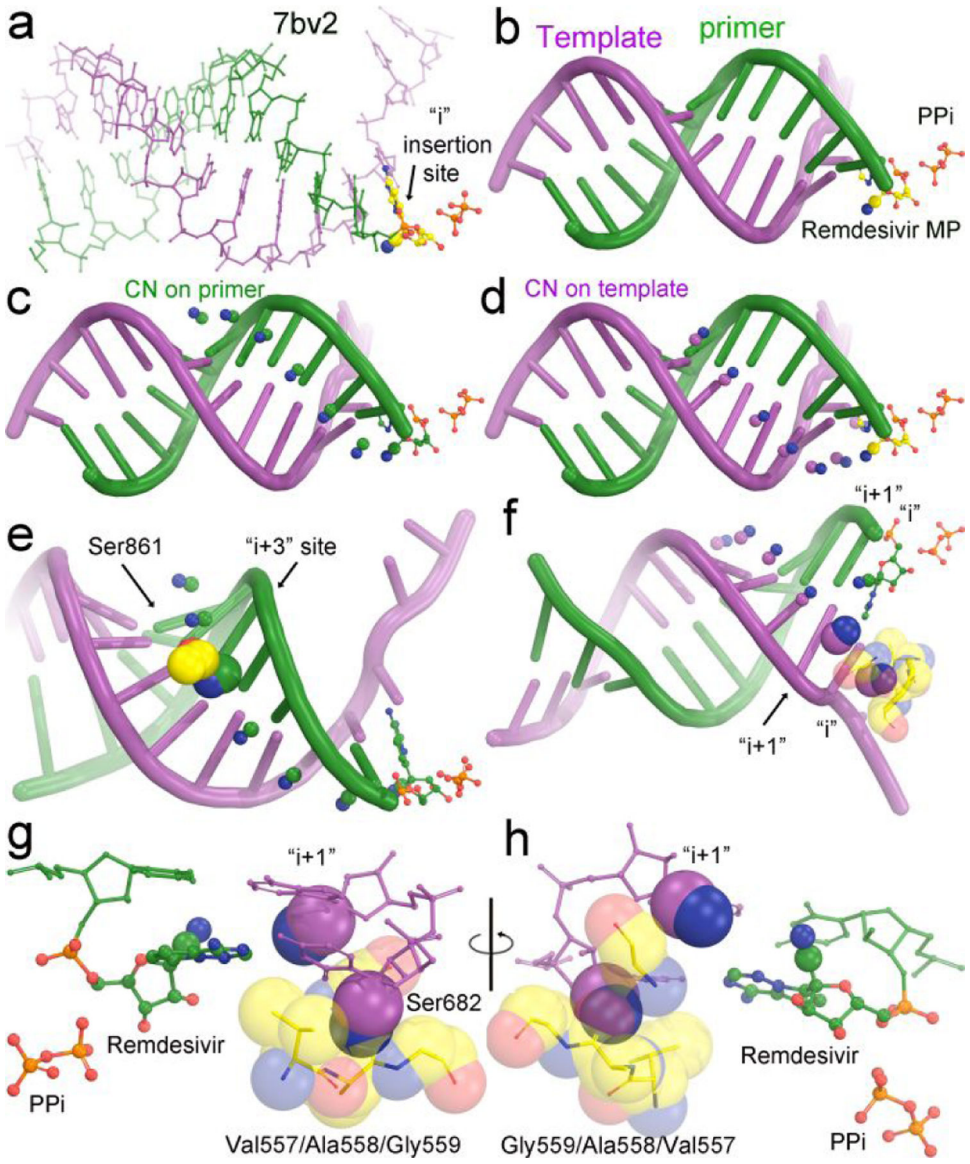

Figure 2.

Modeling of the cyano substitutions at various positions of primer and template nucleotides. (a, b) Experimental structure of the 7bv2 complex with pyrophosphate and RMP in colored ball-and-stick model. The cyano group is shown in 30% of van der Waals radii. (c) Modeling of the cyano substitutions on the nucleotides of the primer strand based on (i) tetrahedral geometry of C1' atom, (ii) the C-C single bond of 1.47 Å, and (iii) the C-N triple bond of 1.14 Å. (d) Modeling of the cyano substitutions on the nucleotides of the template strand. (e) Known clashes between the sidechain of Ser861 of RdRp and the cyano substitution during the translocation from the *i*+3 site to the *i*+4 site. (f) New severe clashes between the cyano substitution of the template nucleotide at the *i* site and backbone carbonyl group of Val557 of RdRp and a minor clash between the cyano substitution of the template nucleotide at the *i*+1 site and the backbone carbonyl of Ser682 of RdRp. (g,h) Close-up views of the first two base pairs in 180° orientation of (f).

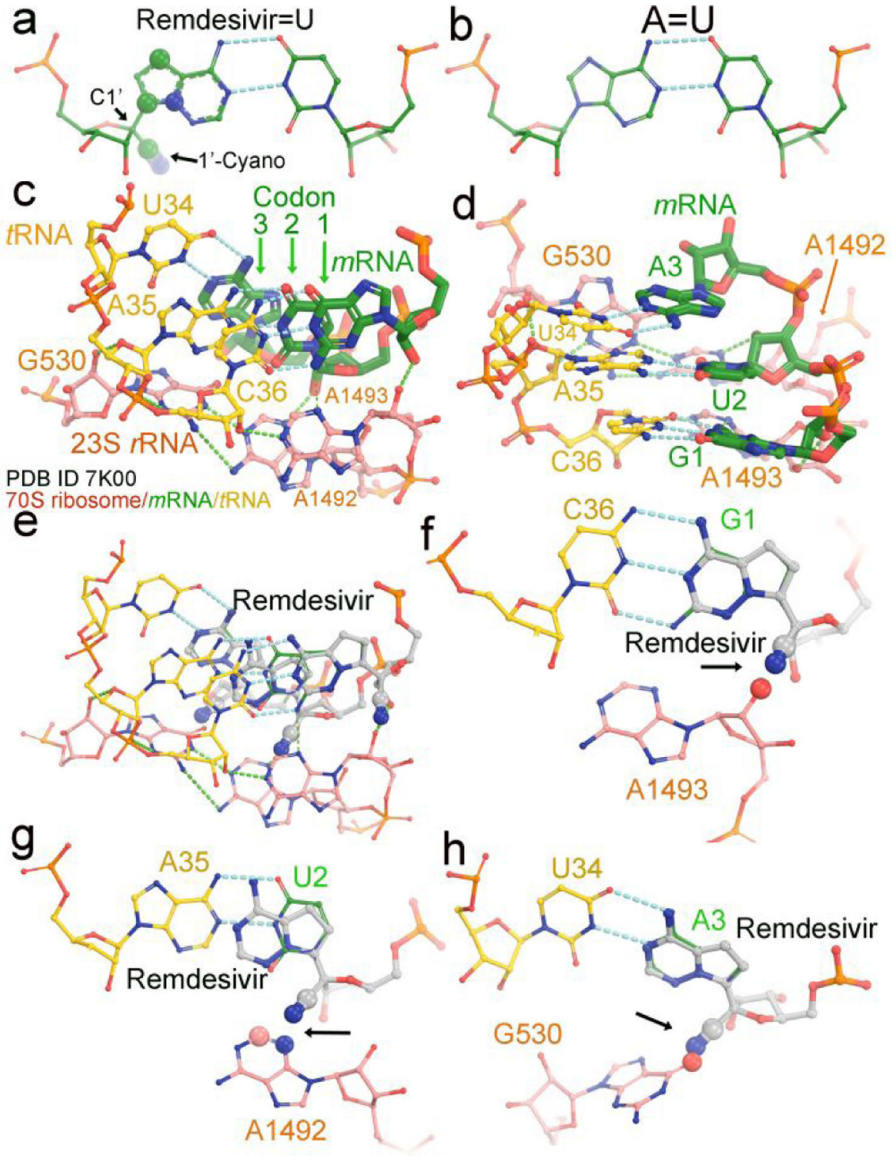

Figure 3.

Modeling of remdesivir on a translating ribosome. (a) Base-pairing between RMP and UMP. The 1'-cyano substitution at C1' and three other substitutions in the nucleobase are in large spheres (30% of van der Waals radii). (b) Watson-Crick AMP=UMP base pair. (c, d) Two views of the translating 70S *E. coli* ribosome cryo-EM structure (PDB ID 7k00) in complex with mRNA (green) and tRNA (yellow) at the decoding center. Three nucleotides of 23S rRNA, G530, A1492 and A1493 are shown in salmon. The three codon nucleotides in the structure are G1, U2 and A3 and in green. Three tRNA anticodon nucleotides are U34, A35 and G36 and in salmon. (e-h) Modeled remdesivir at the first, second, and third codon positions. Large spheres and arrows show where severe clashes occur.