Combination of antiviral drugs inhibits SARS-CoV-2 polymerase and exonuclease and demonstrates COVID-19 therapeutic potential in viral cell culture

Xuanting Wang, Carolina Q. Sacramento, Steffen Jockusch, Otávio Augusto Chaves, Chuanjuan Tao, Natalia Fintelman-Rodrigues, Minchen Chien, Jairo R. Temerozo, Xiaoxu Li, Shiv Kumar, Wei Xie, Dinshaw J. Patel, Cindy Meyer, Aitor Garzia, Thomas Tuschi, Patricia T. Bozza, James J. Russo, Thiago Moreno L. Souza & Jingyue Ju

SARS-CoV-2 has an exonuclease-based proofreader, which removes nucleotide inhibitors such as Remdesivir that are incorporated into the viral RNA during replication, reducing the efficacy of these drugs for treating COVID-19. Combinations of inhibitors of both the viral RNA-dependent RNA polymerase and the exonuclease could overcome this deficiency. Here we report the identification of hepatitis C virus NS5A inhibitors Pibrentasvir and Ombitasvir as SARS-CoV-2 exonuclease inhibitors. In the presence of Pibrentasvir, RNAs terminated with the active forms of the prodrugs Sofosbuvir, Remdesivir, Favipiravir, Molnupiravir and AT-527 were largely protected from excision by the exonuclease, while in the absence of Pibrentasvir, there was rapid excision. Due to its unique structure, Tenofovir-terminated RNA was highly resistant to exonuclease excision even in the absence of Pibrentasvir. Viral cell culture studies also demonstrate significant synergy using this combination strategy. This study supports the use of combination drugs that inhibit both the SARS-CoV-2 polymerase and exonuclease for effective COVID-19 treatment.

1 Center for Genome Technology and Biomolecular Engineering, Columbia University, New York, NY 10027, USA. 2 Department of Chemical Engineering, Columbia University, New York, NY 10027, USA. 3 Laboratory of Immunopharmacology, Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil. 4 National Institute for Science and Technology for Innovation on Diseases of Neglected Population (INCT/IDPN), Center for Technological Development in Health (CDTS), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil. 5 Department of Chemistry, Columbia University, New York, NY 10027, USA. 6 Laboratory on Thymus Research, Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil. 7 National Institute for Science and Technology on Neuroimmunomodulation (INCT/NIM), Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil. 8 Laboratory of Structural Biology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA. 9 Laboratory of RNA Molecular Biology, Rockefeller University, New York, NY 10065, USA. 10 Department of Molecular Pharmacology and Therapeutics, Columbia University, New York, NY 10032, USA. 11 These authors contributed equally: Xuanting Wang, Carolina Q. Sacramento, Steffen Jockusch. 12 These authors jointly supervised this work: Thiago Moreno L. Souza, Jingyue Ju. *Email: tmoreno@cdts.fiocruz.br; dj222@columbia.edu
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for COVID-19, is a positive-sense single-stranded RNA virus. Thus it requires an RNA-dependent RNA polymerase (RdRp) to replicate and transcribe its genome. Because of its large genome (~30 kb) and error-prone RdRp, SARS-CoV-2 also possesses a 3′–5′ exonuclease for proofreading to maintain the integrity of the genome. The replication complex of coronaviruses consists of several viral proteins, including the RdRp itself (nonstructural protein 12; nsp12) and its two accessory proteins (nsp7 and nsp8)4. While a variety of drugs targeting many of the SARS-CoV-2 proteins essential for its infectious cycle have been evaluated, to date no effective antiviral strategy for COVID-19 exists. Remdesivir (RDV), a nucleotide analogue, is the only small-molecule antiviral drug approved by the Food and Drug Administration (FDA) for COVID-19 treatment, although it was shown to have limited benefit in the World Health Organization’s Solidarity Trial. Lack of oral bioavailability and short half-life reduces RDV’s clinical effectiveness. With the appearance of new SARS-CoV-2 variants that pose a threat to public health and may escape the immune response to current vaccines, it is more critical than ever to develop antivirals that can treat COVID-19 effectively. We have investigated a library of nucleotide analogues targeting the SARS-CoV-2 RdRp, several of which have been FDA approved for other viral infections including HIV/AIDS and hepatitis B and C8–11. Of particular interest are those that are able to be incorporated into the replicated RNA by the viral RdRp where they halt or slow further replication. These include, among several others, nucleotide analogues such as Sofosbuvir (Fig. 1a), an immediate terminator of the polymerase reaction which has since entered COVID-19 clinical trials12,13. Remdesivir (Fig. 1b), a delayed terminator14,15, and Tenofovir, an obligate terminator (Fig. 1d)10,11. Recently, we demonstrated that the main domains and the catalytic amino acid residues of the SARS-CoV-2 exonuclease nsp14 interact with the SARS-CoV-2 exonuclease. Since the SARS-CoV-2 RdRp complex, both nucleotide analogues were removed by the SARS-CoV-2 exonuclease complex, although Sofosbuvir was removed at a slower rate16. Currently, three other prodrugs related to nucleotides, Favipiravir (Fig. 1c), Molnupiravir, and AT-527 (Fig. 1e, f), are undergoing clinical trials for COVID-19.

While nucleoside/nucleotide analogues show promising results against SARS-CoV-2, in vitro pharmacological parameters often exceed human plasma exposure. Most likely, nucleoside/nucleotide-based inhibitors are insufficient to block viral RNA replication, because they could be quickly excised by the SARS-CoV-2 exonuclease. A combination of RdRp inhibitors such as Sofosbuvir, Remdesivir, and others listed in Fig. 1 with exonuclease inhibitors may provide a more effective strategy for blocking SARS-CoV-2 RNA replication. We previously described the need to overcome the exonuclease proofreading function in order to completely inhibit SARS-CoV-2 replication in early 2020. An in silico study has proposed combining RdRp and exonuclease inhibitors as a strategy to combat COVID-1917; however, no successful demonstration of such a combined drug strategy has been reported. Hepatitis C virus (HCV) and SARS-CoV-2 are both positive-sense single-stranded RNA viruses. Viruses with small genomes that encode relatively few proteins, such as HCV, must possess multifunctional proteins18. HCV NS5A is a good example because it binds lipids, Zn$^{2+}$ and RNA, as well as participates in phosphorylation and cell signaling events18. Zn$^{2+}$ bound to the NS5A protein coordinates the binding of RNA. The SARS-CoV-2 genome is threefold larger than that of HCV. Although there is no counterpart of NS5A in SARS-CoV-2, the analogous NS5A functions are likely distributed among proteins nsp1 to nsp16 of the coronavirus19. We have previously shown that the NS5A inhibitors Daclatasvir and Velpatasvir inhibit both the SARS-CoV-2 polymerase and exonuclease activities20,21. The combination of Velpatasvir with Remdesivir was shown to have a synergistic effect relative to Remdesivir alone in inhibiting SARS-CoV-2 replication22. Here, we further examined whether the NS5A inhibitors Pibrentasvir, Ombitasvir, and Daclatasvir (structures shown in Supplementary Fig. S-1), possess the anti-exonuclease activity and could synergize with RdRp inhibitors in virus replication assays. We discovered that Pibrentasvir and Ombitasvir have the highest exonuclease inhibitory activity among all the NS5A inhibitors we have tested. As an example, we show here that Pibrentasvir reduced the removal by exonuclease of immediate and delayed RNA chain terminators incorporated into the RNA. Consequently, Pibrentasvir and Ombitasvir synergistically improved the in vitro pharmacological parameters of several RdRp inhibitors, such as Sofosbuvir, Remdesivir, Tenofovir, and Favipiravir. Since all these RdRp inhibitors except Favipiravir, which is approved in Japan for influenza, are FDA approved for various viral infections, candidate combinations of the polymerase and exonuclease inhibitors identified here could be prioritized for advancement to COVID-19 clinical trials.

**Results**

**Molecular docking study of HCV NS5A inhibitors with SARS-CoV-2 exonuclease nsp14.** We performed a molecular docking study to investigate whether the HCV NS5A inhibitors (Supplementary Fig. S-1) interact with the SARS-CoV-2 exonuclease. Since a 3D structure for SARS-CoV-2 nsp14 was not yet available, we built a protein model based on the ortholog in SARS-CoV3. Superposition of the 3D structures of these two enzymes indicates that the main domains and the catalytic amino acid residues of the exonuclease active sites are structurally similar (Supplementary Fig. S-2). The clinically approved HCV NS5A inhibitors are bound to the SARS-CoV-2 nsp14 exonuclease active site with comparable docking scores (Fig. 2c). The exonuclease activity requires the proper coordination of the Mg$^{2+}$ ion with amino acid residues Asp-90, Glu-92, Glu-191, and Asp-273 as well as the RNA substrate. NS5A inhibitors interfere with the coordination among the Mg$^{2+}$ ion, the amino acids in the nsp14 active site and the RNA 3′ terminus, likely preventing nucleotide excision from the RNA (Fig. 2d).

**Inhibition of SARS-CoV-2 exonuclease activity by HCV NS5A inhibitors to prevent the excision of immediate and delayed terminators from RNA.** Using the molecular assays that we have developed previously16, we evaluated if HCV NS5A inhibitors could indeed inhibit SARS-CoV-2 exonuclease activity. We identified Pibrentasvir and Ombitasvir as two strong inhibitors of the SARS-CoV-2 exonuclease. As a proof of principle, we used Pibrentasvir to demonstrate whether such drugs can prevent excision of nucleotide analogues from RNAs terminated with the active forms of prodrugs such as Remdesivir, Sofosbuvir, Tenofovir, Favipiravir, Molnupiravir, and AT-527. These nucleotide analogues are all able to be incorporated into RNA by the SARS-CoV-2 RdRp, where they either block further polymerase extension (Remdesivir, Sofosbuvir, Tenofovir, AT-527) or induce errors in the replicated RNA (Favipiravir, Molnupiravir).

Pibrentasvir and Ombitasvir inhibit the SARS-CoV-2 exonuclease in a concentration-dependent manner, as shown in Fig. 3. RNA (sequence shown at the top of the figure) was incubated with a SARS-CoV-2 pre-assembled exonuclease complex (nsp14/nsp10) at 37 °C for 15 min in the absence (b) and presence of varying amounts of Pibrentasvir (c–e). The RNA (a) and the products of the exonuclease reaction (b–e) were analyzed by MALDI-TOF MS. The peak at 8160 Da corresponds to the intact RNA. In the absence of Pibrentasvir, exonuclease activity caused
nucleotide cleavage from the 3′-end of the RNA as shown by the seven shorter fragments of lower molecular weight corresponding to stepwise cleavage of 1–7 nucleotides (b), with only ~7% intact RNA remaining (peak at 8167 Da). Pibrentasvir (1, 10, and 20 μM) reduced exonuclease activity in a concentration-dependent manner as shown by the reduced intensities of the fragmentation peaks and more prominent intact RNA peak (c–e). Ombitasvir displayed similar efficiency of exonuclease activity inhibition (Fig. 3f–i) as Pibrentasvir. Daclatasvir also exhibits inhibition of exonuclease activity, but at higher concentrations than either Pibrentasvir or Ombitasvir (Supplementary Fig. S-3).

We next determined if RNA terminated with the active forms of prodrugs such as Sofosbuvir, Remdesivir, Favipiravir, Molnupiravir, and AT-527 were also less likely to be excised by SARS-CoV-2 exonuclease in the presence of Pibrentasvir. We generated Sofosbuvir and Remdesivir terminated RNA by single-base extension of RNA template-loop-primers with the active triphosphate forms of Sofosbuvir and Remdesivir, respectively, using the replication complex assembled from SARS-CoV-2 nsp12 (the viral RdRp) and nsp7 and nsp8 proteins (RdRp cofactors)11,16. The Sofosbuvir (S)-terminated RNA and Remdesivir (R)-terminated RNA (sequences shown at the top of Fig. 4) were incubated with the SARS-CoV-2 nsp14/nsp10 complex at 37 °C for 15 min in the absence (Fig. 4b, e) and presence of 20 μM Pibrentasvir (c, f). MALDI-TOF MS analysis of the intact RNAs (a, d) and the products of the exonuclease reactions (b, c, e, f) was performed. In the absence of Pibrentasvir, exonuclease activity caused nucleotide cleavage from the 3′-end of the RNA as shown by the seven shorter fragments of lower molecular weight corresponding to stepwise cleavage of 1–7 nucleotides (b, e), with a comparable reduction in the intact RNA (8178 Da for S-terminated RNA or 8209 Da for R-terminated RNA). When 20 μM Pibrentasvir was added, exonuclease activity was substantially inhibited as shown by the reduced intensities of the fragmentation peaks and increased peak heights of the intact RNAs (c, f). Thus, the SARS-CoV-2 exonuclease activity is significantly inhibited by Pibrentasvir to prevent nucleotide excision from both Sofosbuvir and Remdesivir terminated RNA. Similarly, Pibrentasvir substantially reduced excision of the nucleotide analogues derived from Favipiravir (Supplementary Fig. S-4), Molnupiravir (NHC, N4-hydroxycytidine, Supplementary Fig. S-5) and AT-527 (Supplementary Fig. S-6) from the 3′ terminus of RNA. In terms of base pairing, Favipiravir-RTP acts like a G or A23, NHC-TP behaves like a C or U24 and AT-9010 (the active triphosphate form of AT-527) is a G analogue25. As expected, Favipiravir-RTP was incorporated into RNA opposite C.
Fig. 2 3D representation of the best docking poses for HCV NS5A inhibitors in the nsp14 exonuclease active site. The NS5A inhibitors were built and minimized in terms of energy by density functional theory (DFT). Docking was performed using GOLD 2020.2 software with ChemPLP as scoring function. Because of the diversity in NS5A inhibitors’ molecular weights, they were allowed to dock within 8 or 10 Å spheres in the SARS-CoV-2 nsp14 exonuclease active site, constructed in Supplementary Fig. S-2. The NS5A and catalytic amino acid residues are in stick representation. Mg$^{++}$ and Zn$^{++}$ are represented as green and indigo blue spheres, respectively.

| Antiviral   | 8 Å    | 10 Å   |
|------------|--------|--------|
| Ombitasvir | 20.36  | 62.64  |
| Elbasvir   | 55.38  | 60.83  |
| Ledipasvir | 12.14  | 57.78  |
| Velpatasvir| 16.33  | 57.29  |
| Daclatasvir| 18.41  | 49.25  |
| Pibrentasvir| 17.89 | 47.12  |

SARS-CoV-2 susceptibility to inhibition by immediate/delayed RNA chain terminators and error-prone nucleotide analogues is enhanced in Calu-3 cells by HCV NS5A inhibitors that inhibit the exonuclease. The prodrugs of RdRp inhibitors are activated by cellular enzymes to the active triphosphates. Therefore, the above enzymatic results need to be further evaluated at the cellular level, as interference in any of the activation pathways may impact the synergistic inhibitory activity described in the enzymatic assays. We evaluated if SARS-CoV-2’s in vitro susceptibility to clinically approved RdRp prodrugs could be enhanced by combining them with HCV NS5A inhibitors, which were shown to inhibit the exonuclease activity above. Calu-3 cells, which recapitulate the type II pneumocytes, cells destroyed in the course of severe COVID-19, were infected at a multiplicity of infection (MOI) of 0.01. The experiment was performed with natural RNA, which produced a peak of the intact RNAs (f, 9807 Da). In Fig. 5a, a comparison experiment was performed with natural RNA, which produced essentially identical results. Therefore, these results indicated that the SARS-CoV-2 exonuclease activity is also significantly inhibited by Pibrentasvir to prevent nucleotide excision from RNA with delayed termination by Remdesivir.

We previously showed that Tenofovir diphosphate (Tfv-DP) can be incorporated into RNA as an obligate terminator by the SARS-CoV-2 RdRp complex. As an acyclic nucleotide, Tfv-DP lacks a normal sugar ring configuration, and thus we reasoned that it is less likely to be recognized by SARS-CoV-2 exonuclease. Therefore, we investigated whether Tfv can be removed by the exonuclease from the extended RNA. As shown in Fig. 6, Tfv-DP was incorporated into an RNA loop primer to produce the Tfv-terminated RNA. When incubation with the SARS-CoV-2 nsp14/nsp10 complex was carried out at 37 °C for 15 min, minimal cleavage was observed for Tfv-terminated RNA (e) relative to RNA extended with a natural A nucleotide (b), where there was substantial cleavage. When 20 µM Pibrentasvir was added, exonuclease activity was almost completely eliminated as shown by almost no fragmentation peaks and the essentially intact RNA peak (f). To directly compare the exonuclease resistance activities, we incubated both the RNA terminated with Tfv-DP and the natural RNA terminated with the A nucleotide in the same reaction tube, and compared the patterns obtained by MALDI-TOF MS (Supplementary Fig. S-7). Because these two RNAs have different molecular weights, it was straightforward to determine whether the resulting cleavage products were derived from the Tfv- or the A-terminated RNA. The results shown in Fig. 6 and Supplementary Fig. S-7 demonstrate that Tenofovir, once incorporated into RNA, is mostly resistant to excision by the SARS-CoV-2 exonuclease.
infection (MOI) of 0.1 and treated with the drugs under investigation alone or in combination. After 2–3 days, culture supernatant was harvested and titered in Vero E6 cells to quantify the infectious viruses as plaque-forming units (PFU). The NS5A inhibitors Pibrentasvir, Ombitasvir, and Daclatasvir inhibited SARS-CoV-2 replication in a dose-dependent manner (Supplementary Fig. S-8), but with lower potencies than Remdesivir (RDV) (Supplementary Table S-1). Nevertheless, anti-SARS-CoV-2 potencies of the HCV NS5A inhibitors were ~10 times higher (EC_{50} of 0.7 μM for Pibrentasvir and 0.4 μM for Ombitasvir) compared to other repurposed RdRp inhibitors, such as Sofosbuvir (6.2 μM), Tenofovir (4.3 μM) and Favipiravir (7.8 μM) (Supplementary Table S-1).

To produce clinical benefit, inhibition of SARS-CoV-2 exonuclease by NS5A inhibitors need to enhance the anti-coronavirus activity of RdRp inhibitors by orders of magnitude. Thus, Sofosbuvir...
and Tenofovir (immediate terminators), RDV (a delayed chain terminator), and Favipiravir (an error-prone nucleobase) were tested at various concentrations along with the EC25 of Pibrentasvir (0.1 μM), Ombitasvir (0.1 μM) and Daclatasvir (0.5 μM) and the results are listed in Table 1, which shows the resulting EC50, EC90, and EC99 values for the RdRp inhibitors in the presence and absence of the exonuclease inhibitors. Figure 7 is a log-log plot of the concentration of RdRp inhibitor alone (or in combination with exonuclease inhibitors) vs virus production in plaque-forming units. Supplementary Fig. S-9 shows the same data using a semi-log plot of inhibitor concentration vs percent viral inhibition, for easier visualization of the inhibitor effects. Pibrentasvir improved RDV’s efficacy at the 90% and 99% levels six- and threefold, respectively (Table 1). Moreover, Pibrentasvir improved the potency of Favipiravir and Tenofovir tenfold at EC50, but only modestly increased RDV’s efficacy at EC90 (Table 1). In the presence of Daclatasvir, Favipiravir’s virus inhibitory efficacy at the 90% level was achievable (Table 1).

To further determine if the combination of RdRp and NS5A inhibitors result in synergistic or additive effects in inhibiting SARS-CoV-2 replication, we generated combined concentration curves with these two categories of antivirals under the same experimental conditions described above. The virus inhibition results were analyzed using SynergyFinder 2.0 software, which represents synergistic, additive, and antagonistic effects as scores above 10, between 10 and $-10$, and below $-10$, respectively. The results show that Ombitasvir displayed synergistic effects with RDV (Fig. 8a), Sofosbuvir (Fig. 8b), Tenofovir (Fig. 8c), and Favipiravir (Fig. 8d). Pibrentasvir was synergistic with RDV and Favipiravir (Fig. 8a, d), whereas it displayed additive effects with Sofosbuvir and Tenofovir (Fig. 8b, c). Daclatasvir displayed an additive effect with RDV (Fig. 8a) and synergistic effects with the other RdRp inhibitors tested (Fig. 8b–d). These results demonstrate that the exonuclease inhibitory effect of NS5A inhibitors consistently enhanced the potency of prodrugs of the nucleotide analogues in the cell culture system.

**Fig. 4 Inhibition of SARS-CoV-2 exonuclease activity by Pibrentasvir for Sofosbuvir (S) and Remdesivir (R) terminated RNA.** A mixture of 500 nM RNAs (sequences shown at the top of the figure) and 50 nM SARS-CoV-2 pre-assembled exonuclease complex (nsp14/nsp10) were incubated in buffer solution at 37 °C for 15 min in the absence (b, e) and presence of 20 μM Pibrentasvir (c, f). The intact RNAs (a, d) and the products of the exonuclease reactions (b–f) were analyzed by MALDI-TOF MS. The signal intensity was normalized to the highest peak. In the absence of Pibrentasvir, exonuclease activity caused nucleotide cleavage from the 3′-end of the RNA as shown by the lower molecular weight fragments corresponding to cleavage of 1-7 nucleotides (b, e). When 20 μM Pibrentasvir was added, exonuclease activity was reduced as shown by the reduced intensities of the fragmentation peaks and increased intact RNA peaks (c, f).
Discussion

We previously demonstrated that the FDA-approved HCV NS5A inhibitors, Daclatasvir and Velpatasvir, and to a lesser extent the NS5A inhibitors Elbasvir and Ledipasvir, can inhibit the SARS-CoV-2 exonuclease 20,21. Here, we showed that two additional NS5A inhibitors, Pibrentasvir and Ombitasvir, also inhibit the exonuclease, and have the highest inhibitory activity based on our molecular assay. These compounds are predicted to interfere with the binding of the Mg$^{2+}$ ion with the 3′-terminus of the RNA in the active site of the exonuclease (nsp14). The Mg$^{2+}$ ion coordinates with amino acid residues Asp-90, Glu-92, Glu-191, and Asp-273 and the 3′-terminus of the RNA. Because the NS5A inhibitors interfere with this coordination, they are likely to prevent nucleotide excision from the RNA (Fig. 2). A recent in silico modeling study has suggested that Ritonavir also binds to the active site of nsp14, which led the authors to the prediction that Ritonavir may inhibit exonuclease activity31. We have experimentally shown (Supplementary Fig. S-10) that Ritonavir and Lopinavir, HIV protease inhibitors that make up the combination drug Kaletra, inhibit the SARS-CoV-2 exonuclease in a concentration-dependent manner, but with less potency than Pibrentasvir and Ombitasvir. Kaletra has gone through extensive clinical trials for COVID-19 but shows limited efficacy by itself32,33. Recently, using a digital drug development approach combining artificial intelligence and experimental validation to screen drug combinations for potential combination therapy against SARS-CoV-2, it was determined that one of the most effective potential combinations was Remdesivir, Ritonavir, and Lopinavir34. This synergy of Ritonavir and Lopinavir with Remdesivir may be due to the exonuclease inhibitory activity of these two drugs (Supplementary Fig. S-10).

NS5A inhibitors enhance the antiviral activity of RDV, Tenofovir, Sofosbuvir, and Favipiravir, likely due to their inhibition of exonuclease activity. The combinations of drugs described here, especially the orally available antivirals, have the potential to progress to COVID-19 clinical trials. We show that by combining Pibrentasvir or Ombitasvir with Remdesivir, Sofosbuvir, Tenofovir, or Favipiravir, higher inhibitory activity for SARS-CoV-2 was achievable at lower doses, bringing the nucleotides' pharmacological parameters more in line with their pharmacokinetic exposures (https://pdf.hres.ca/dpd_pm/00040336.PDF)28,35. The nucleotide inhibitors Remdesivir and Sofosbuvir are incorporated into the replicating RNA to inhibit further polymerase reaction, but they are rapidly removed by the exonuclease in the absence of exonuclease inhibitors such as Pibrentasvir and Ombitasvir. However, in the presence of these inhibitors, any nucleotide inhibitors incorporated into the RNA will not be rapidly excised by the exonuclease and their inhibitory effect on overall viral RNA replication will be substantially enhanced. Recently, the coronavirus exonuclease has also been shown to promote viral recombination which can increase the emergence of novel strains36; providing additional incentive for targeting this enzyme with antivirals.

![Fig. 5 Inhibition of SARS-CoV-2 exonuclease activity by Pibrentasvir for natural RNA and RNA with delayed termination by Remdesivir (R).](image-url)
Previously, Baddock et al. screened a series of molecules for SARS-CoV-2 exonuclease activity, including Ebselen and Disulfiram, with Ebselen having the higher inhibitory activity. Ebselen and Disulfiram have multiple additional SARS-CoV-2 protein targets and Chen et al. used a combination of Ebselen/Disulfiram with Remdesivir to demonstrate a small synergistic inhibitory effect on viral replication. Recently, it has been reported that HCV nsp3/4A protease inhibitors that inhibit the SARS-CoV-2 papain-like protease synergize with Remdesivir to suppress viral replication in cell culture. In our investigation, by focusing on combinations of clinically approved drugs used during routine treatment of HIV/HCV/influenza-infected individuals, we discovered significant synergistic combinations of some of these drugs that inhibit both the SARS-CoV-2 polymerase and exonuclease activities.

Favipiravir (6-fluoro-3-hydroxy-2-pyrazinocarboxamide, Fig. 1c), a drug used to treat influenza, has been investigated for the treatment of COVID-19. It has a modified nucleobase that can base pair with both C and U. Favipiravir is converted to the active triphosphate form, Favipiravir-ribofuranosyl-5’-triphosphate (Favipiravir-RTP), by a cellular enzyme, hypoxanthine guanine phosphoribosyltransferase, and cellular kinases (Supplementary Fig. S-11). Favipiravir-RTP acts as a viral RNA polymerase inhibitor, with great potential in the treatment of a wide variety of RNA virus infections, including different strains of influenza. Favipiravir-RTP is incorporated into the growing RNA chain by the SARS-CoV-2 RdRp complex and causes C-to-U and G-to-A transitions. In a more recent study, Favipiravir was found to exhibit antiviral activity against SARS-CoV-2 via a combination of mechanisms, including slowing RNA synthesis, initiation of delayed chain termination, and lethal mutagenesis.

The 3D structure of the SARS-CoV-2 RdRp complex (nsp12, nsp7, and nsp8) with the RNA substrate and Favipiravir-RTP was determined, providing insight into the mode of action of this drug. Here, we demonstrated that the excision of Favipiravir nucleotide from the 3’ end of RNA by the SARS-CoV-2 exonuclease complex is greatly reduced in the presence of Pibrentasvir (Supplementary Fig. S-4). Since Pibrentasvir and Ombitasvir inhibited the SARS-CoV-2 exonuclease proofreading activity, when these drugs are combined with Favipiravir, it is more likely that Favipiravir-induced mutations will persist and jeopardize viral replication. This is supported by our cell culture virus inhibition data indicating that the combination of Favipiravir with Ombitasvir resulted in a 2-log10 inhibition, reducing virus replication by 99% (Table 1) and that NS5A inhibitors synergized with Favipiravir (Fig. 8).

Fig. 6 Inhibition of SARS-CoV-2 exonuclease activity by Pibrentasvir for natural RNA and Tenofovir (Tfv) terminated RNA. A mixture of 500 nM RNAs (sequences shown at the top of the figure) and 50 nM SARS-CoV-2 pre-assembled exonuclease complex (nsp14/nsp10) were incubated in buffer solution at 37 °C for 15 min in the absence (b, e) and presence of 20 μM Pibrentasvir (c, f). The intact RNAs (a, d) and the products of the exonuclease reactions (b–f) were analyzed by MALDI-TOF MS. The signal intensity was normalized to the highest peak. In the absence of Pibrentasvir, exonuclease activity caused nucleotide cleavage from the 3’-end of the natural RNA as shown by the lower molecular weight fragments corresponding to cleavage of 1-8 nucleotides (b). However, for Tfv-terminated RNA, only minor cleavage was observed (e). When 20 μM Pibrentasvir was added, exonuclease activity was reduced as shown by the reduced intensities of the fragmentation peaks and the increased peak height of the intact RNA (c, f).

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N4-hydroxycytidine (NHC, Fig. 1e) has been shown to inhibit SARS-CoV-2 and other coronaviruses in mice and human airway epithelial cells 24. NHC is incorporated opposite either A or G in the template strand. Molnupiravir, which is activated to the triphosphate form (NHC-TP) by cellular enzymes, is currently in a COVID-19 clinical trial 46. Here, we showed that the excision of NHC from the 3′ end of RNA by the SARS-CoV-2 exonuclease complex is substantially reduced in the presence of Pibrentasvir (Supplementary Fig. S-5).

Table 1 In vitro pharmacological parameters of potency and efficacy of combinations of RdRp and HCV NS5A inhibitors on SARS-CoV-2 replication in Calu-3 cells.

| Drug | EC50 Mean ± SEM | EC90 Mean ± SEM | EC99 Mean ± SEM |
|------|-----------------|-----------------|-----------------|
| Remdesivir (RDV) | 0.09 ± 0.002 | 0.4 ± 0.03 | 1.1 ± 0.2 |
| Sofosbuvir | 6.2 ± 0.3 | ND | ND |
| Tenofovir | 4.3 ± 2.1 | ND | ND |
| Favipiravir | 7.8 ± 1.2 | ND | ND |
| Pibrentasvir | 0.7 ± 0.2 | 4.2 ± 0.6 | ND |
| RDV + Pibrentasvir (0.1 μM) | 0.008* ± 0.0009 | 0.07* ± 0.03 | 0.3* ± 0.09 |
| Sofosbuvir + Pibrentasvir (0.1 μM) | 8.3 ± 0.5 | ND | ND |
| Tenofovir + Pibrentasvir (0.1 μM) | 0.5* ± 0.05 | 8* ± 1.5 | ND |
| Favipiravir + Pibrentasvir (0.1 μM) | 0.5* ± 0.03 | 8* ± 0.5 | ND |
| Ombitasvir | 0.4 ± 0.05 | 3.3 ± 0.5 | ND |
| RDV + Ombitasvir (0.1 μM) | 0.008* ± 0.0003 | 0.01* ± 0.05 | 0.5* ± 0.2 |
| Sofosbuvir + Ombitasvir (0.1 μM) | 6 ± 1.3 | 9* ± 5 | ND |
| Tenofovir + Ombitasvir (0.1 μM) | 0.8* ± 0.07 | 7* ± 1.6 | 8.9* ± 0.4 |
| Favipiravir + Ombitasvir (0.1 μM) | 0.15* ± 0.04 | 8* ± 0.4 | 9.5* ± 0.4 |
| Daclatasvir | 0.7 ± 0.08 | 3.8 ± 1.2 | ND |
| RDV + Daclatasvir (0.5 μM) | 0.008* ± 0.0006 | 0.1* ± 0.06 | 0.5* ± 0.1 |
| Sofosbuvir + Daclatasvir (0.5 μM) | 0.6* ± 0.05 | 9* ± 0.2 | ND |
| Tenofovir + Daclatasvir (0.5 μM) | 0.01* ± 0.004 | 6* ± 1.2 | 7.5* ± 0.5 |
| Favipiravir + Daclatasvir (0.5 μM) | 0.12* ± 0.05 | 8* ± 0.5 | ND |

*P < 0.05 comparing the in vitro pharmacological parameters of the RNA polymerase inhibitor alone vs its association with an HCV NS5A inhibitor using the Student’s t test.

Fig. 7 Antiviral activity of combinations of SARS-CoV-2 polymerase and exonuclease inhibitors. Calu-3 cells, at a density of 5 × 10^5 cells/well in 48-well plates, were infected with SARS-CoV-2 at a MOI of 0.1, for 1 h at 37 °C. An inoculum was removed and cells were washed and incubated with fresh DMEM containing 10% FBS and the indicated concentration of Remdesivir (RDV) (a), Sofosbuvir (b), Tenofovir (c), and Favipiravir (d), alone and in combination with the HCV NS5A inhibitors. Supernatants were assessed after 48–72 h. Viral replication in the culture supernatant was measured as PFU/mL by titrating in Vero E6 cells. Results are displayed as virus titers. The data represent means ± SEM of three independent experiments.
Fig. 8 Synergistic effects of combinations of RdRp and NS5A inhibitors in inhibiting SARS-CoV-2 replication. Calu-3 cells (5 x 10^5 cell/well) were infected with SARS-CoV-2 at a MOI of 0.1. After a 1 h of inoculation period, cells were treated with Remdesivir (RDV) (a), Sofosbuvir (SFV) (b), Tenofovir (TFV) (c), or Favipiravir (d) (FPV) with or without Pibrentasvir (PIB), Ombitasvir (OMB), or Daclatasvir (DCV); culture supernatant was harvested 2-3 days post-infection and titered in Vero E6 cells by PFU. Results were calculated as the percentage of inhibition and plotted online using SynergyFinder2.0 (https://synergyfinder.fimm.fi/). Scores were determined after applying the highest single agent (HSA) algorithm. Scores are interpreted as follows: synergistic effect >10, additive effect <10 and >−10, antagonistic effect <−10.
AT-57 (Fig. 1f), a prodruk of a guanosine nucleotide analogue with both the base and phosphate group masked, is converted by cellular enzymes to the active triphosphate form, 2'-fluoro-2'-
methyl guanosine-5'-triphosphate (AT-9010, Gfm

The active triphosphate form of AT-57 is structurally similar to that of Sofosbuvir with the only difference being that it has a guanosine base in place of uracil. AT-57 inhibits SARS-CoV-2 by targeting both the RdRp activity and the Nido

The combination of NS5A inhibitors with Tenofovir protected the Tenofovir-terminated RNA from excision by exonuclease activity. The combination of NS5A inhibitors with Tenofovir also substantially increased the virus inhibition efficiency in cell culture (Table 1 and Fig. 8).

Our results from the molecular enzymatic assays and the virus cell culture inhibitory data both indicate that the use of inhibitors of the viral RdRp and the exonuclease proofreader as a combination therapy is expected to have enhanced efficacy in treating COVID-19. This combination approach may allow two-log

The RdRp of SARS-CoV-2, referred to as nsp12, and its two protein cofactors, nspt7, and nsp8, shown to be required for the processive polymerase activity of nsp12, were cloned and purified as described1.10 The 3'-exonuclease, referred to as nsp14, and its protein cofactor, nsp10, were cloned and expressed based on the SARS-CoV-2 genome sequence. Kemdesvir (RDV-TP) and NHC-TP produces the active triphosphate form for RDV-TP extension; 5'-UUUU-CAUCGGCGAGUUGUUCUACGGG-3' for RDV-TP extension; 5'-UUCUUCAUCGGCGAGUUGUUCUACGGG-3' for SOF-TP extension) in 1 × RdRp reaction buffer was added to the annealed RNA template-loop-primer solution and incubated for an additional 10 min at room temperature. Finally, 5 µL of a solution containing 0.2 mM RDV-TP or 2 mM SOF-TP in 1 × reaction buffer was added, and incubation was carried out for 2 h at 30 °C. The final concentrations of reagents in the extension reactions were 2 µM nsp2/nsp7, 5 µM RNA template-loop-primer, 50 µM RDV-TP, and 500 µM SOF-TP. The 1 × reaction buffer contains the following reagents: 10 mM Tris-HCl pH 8, 100 mM MgCl2, and 1 mM β-mercaptoethanol. Desalting of the reaction mixture was performed with an Oligo Clean & Concentrator kit (Zymo Research) resulting in ~10 µL purified aqueous RNA solutions. 1 µL of each solution was subjected to MALDI-TOF MS (Bruker ultraFlextreme) analysis. The remaining ~9 µL extended template-loop-primer solutions were used to test exonuclease activity.

Extension reactions with SARS-CoV-2 RNA-dependent RNA polymerase complex to produce Remdesivir (RDV) and Sofosbuvir (SOF) terminated RNAs. In total, 10 µL of 10 µM RNA template-loop-primers (5'-UUUU-CAUCGGCGAGUUGUUCUACGGG-3' for RDV-TP extension; 5'-UUCUUCAUCGGCGAGUUGUUCUACGGG-3' for SOF-TP extension) in 1 × RdRp reaction buffer was added to the annealed RNA template-loop-primer solution and incubated for an additional 10 min at room temperature. Finally, 5 µL of a solution containing 0.2 mM RDV-TP or 2 mM SOF-TP in 1 × reaction buffer was added, and incubation was carried out for 2 h at 30 °C. The final concentrations of reagents in the extension reactions were 2 µM nsp2/nsp7, 5 µM RNA template-loop-primer, 50 µM RDV-TP, and 500 µM SOF-TP. The 1 × reaction buffer contains the following reagents: 10 mM Tris-HCl pH 8, 100 mM MgCl2, and 1 mM β-mercaptoethanol. Desalting of the reaction mixture was performed with an Oligo Clean & Concentrator kit (Zymo Research) resulting in ~10 µL purified aqueous RNA solutions. 1 µL of each solution was subjected to MALDI-TOF MS (Bruker ultraFlextreme) analysis. The remaining ~9 µL extended template-loop-primer solutions were used to test exonuclease activity.

Extension reactions with Therminor II polymerase to produce AT-9010 terminated RNA. The RNA template-loop primer (5'-UUUUUC CCCCGGGUAGUUUUCUACGGG-3') was annealed at 75 °C for 3 min and cooled to room temperature for 25 min in 1× ThermoPol buffer before adding the other ingre

Expression and purification of the SARS-CoV-2 exonuclease nsp14/nsp10 complex. The 3'-exonuclease, referred to as nsp14, and its protein cofactor, nsp10, were cloned and expressed based on the SARS-CoV-2 genome sequence. The plasmid encoding the final 20 µL extension reaction mixture containing 5 µM of the RNA template-loop-primer, 250 µM, 500 µM or 1 mM AT-9010, 2 mM MCoT2 and 0.2 unit Therminor II in 1× ThermoPol buffer was incubated in a thermal cycler using the following protocol (28 cycles of 45 °C for 30 s, 55 °C for 30 s, 65 °C for 30 s). Desalting of the reaction mixture was performed with an Oligo Clean & Concentrator kit (Zymo Research) resulting in ~10 µL purified aqueous RNA solutions. 1 µL of each solution was subjected to MALDI-TOF MS (Bruker ultraFlextreme) analysis. The remaining ~9 µL extended template-loop-primer solutions were used to test exonuclease activity.

Extension reactions with SARS-CoV-2 RNA-dependent RNA polymerase to produce Favipiravir or NHC terminated RNAs. In all, 10 µL of 10 µM RNA template-loop-primers (5'-UUUUUC CCCCGGGUAGUUUUCUACGGG-3' for Fav-RTC extension; 5'-UUUUUC CCCCGGGUAGUUUUCUACGGG-3' for Fav-RTC extension) in 1 × RdRp reaction buffer was added to the annealed RNA template-loop-primer solution and incubated for an additional 10 min at room temperature. Finally, 5 µL of a solution containing ~2 mM Fav-RTC or 0.4 mM NHC in 1 × reaction buffer was added and incubation was carried out for 3 h at 30 °C. The final concentrations of reagents in the extension reactions were 2 µM nsp2/nsp7, 5 µM RNA template-loop-primer, 250 µM Fav-RTC or 100 µM NHC-TP. The 1 × reaction buffer contains the following reagents: 10 mM Tris-HCl pH 8, 100 mM MgCl2 and 1 mM β-mercaptoethanol.
Desalting of the reaction mixture was performed with an Oligo Clean & Concentrator kit (Zymo Research) resulting in ~10 µL purified aqueous RNA solutions. In all, 5 µL of a solution containing 80 µM GTP and 80 µM UTP in 1× reaction buffer was subjected to MALDI-TOF MS (Bruker ultraflXtreme) analysis. The remaining ~9 µL extended template-loop-primer solutions were used to test exonuclease activity.

**Extension reactions with reverse transcriptase to produce Tenofovir (Tfv) terminated RNAs.** The RNA template-loop primer (5′-UUUUUUCAGCGCCAGUUGUUAAAGCCG-3′) was annealed at 75 °C for 3 min and cooled to room temperature before adding the remaining ingredients. The final 20 µL reaction mixture containing 5 µM of the RNA template-loop primer, 500 µM Tenofovir-DP and 200 units Superscript IV Reverse Transcriptase in 1× reaction buffer (50 mM Tris-HCl (pH 8.3), 4 mM MgCl₂, 10 mM DTT, 50 mM KCl) was incubated at 45 °C for 3 h. Desalting of the reaction mixture was performed with an Oligo Clean & Concentrator kit (Zymo Research) resulting in ~10 µL purified aqueous RNA solutions. In total, 1 µL of each solution was subjected to MALDI-TOF MS (Bruker ultraflXtreme) analysis. The remaining ~9 µL extended template-loop-primer solutions were used to test exonuclease activity.

**Extension reactions with SARS-CoV-2 RNA-dependent RNA polymerase to produce Remdesivir (RDV) delayed terminated RNA.** In all, 10 µL of 10 µM RNA template-loop-primers (5′-UUUUUUCAGCGCCAGUUGUUAAAGCCG-3′ for RDV-TP + UTP extension) in 1 × RdRp reaction buffer was first annealed by heating to 75 °C for 3 min and cooling to room temperature. In total, 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. In total, 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature. 5 µL of 8 µM RdRp complex (nsp12/nsp7/nsp8) in 1× RdRp reaction buffer was annealed by heating to 75 °C for 3 min and cooling to room temperature.

**Molecular docking procedure.** The chemical structures for Pibrentasvir, Ombitasvir, Dasbasantivir, Ledipasvir, and Velpatasvir (Supplementary Fig. S-1) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and minimized in terms of energy by Density Functional Theory (DFT), with the Becke-3-Lee-Yang Parr (B3LYP) method and the standard 6-31G* basis set available in Spartan’18 software (Wavefunction, Inc., Irvine, USA) (https://www.wavefun.com/). Since the 3D structure for the SARS-CoV-2 RNA-dependent RNA polymerase was not available, a structural model was built via the online tool Swiss Model software (University of Basel, Basel, Switzerland) (https://swissmodel.expasy.org/) using the crystallographic structure of SARS-CoV-14 as a template (Protein Data Bank (PDB) code: 5CFT) and the 3D structure of SARS-CoV-2 RNA-dependent RNA polymerase. The best-modelled structure was chosen according to the Quantitative Model Quality Evaluation (QMEAN) and Global Model Quality Estimation (GOMO) values.

**Cells and virus.** African green monkey kidney cells (Vero, subtype E6) and the human lung epithelial cell line (Calu-3) cells were cultured in high glucose DMEM and low glucose DMEM medium, both complemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂. SARS-CoV-2 was prepared in Vero E6 cells at a MOI of 0.01. Originally, the isolate was obtained from a nasopharyngeal swab from a confirmed case in Rio de Janeiro, Brazil (GenBank #MT710174; Institutional Review Board approval, 30650420.4.1001.0008). All procedures related to virus culture were handled in a Biosafety Level 3 (BSL3) multiuser facility according to WHO guidelines. Virus titers were determined as PFU/mL. Virus stocks were kept in −80°C freezers.

**Cytotoxicity assay.** Monolayers of 1.5 × 10⁴ cells in 96-well plates were treated for 3 days with various concentrations of drugs (from 1000 to 10 µM) of the antiviral drugs. Then, 5 mg/ml 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in DMEM was added to the cells in the presence of 5% CO₂. SARS-CoV-2 was prepared in Vero E6 cells at a MOI of 0.01. Originally, the isolate was obtained from a nasopharyngeal swab from a confirmed case in Rio de Janeiro, Brazil (GenBank #MT710174; Institutional Review Board approval, 30650420.4.1001.0008). All procedures related to virus culture were handled in a Biosafety Level 3 (BSL3) multiuser facility according to WHO guidelines. Virus titers were determined as PFU/mL. Virus stocks were kept in −80°C freezers. To a 10 µL solution of 100 nM exonuclease complex (nsP4/10) in 1× exonuclease reaction buffer, 10 µL of annealed RNA mixture (1 µM each) was added and incubated at 37°C for 30 min. The final concentrations of reagents in the 20 µL reaction were: 50 nM nsP4/10, 8 µM Tspo, 20 µM Pibrentasvir, 500 nM DMSO. The 1× exonuclease reaction buffer contains the following reagents: 40 mM Tris-HCl pH 8, 1,5 mM MgCl₂, and 5 mM DTT. After incubation for 15 min, each reaction was quenched by adding 2.2 µL of an aqueous solution of EDTA (100 mM). Following desalting using an Oligo Clean & Concentrator kit (Zymo Research), the samples were subjected to MALDI-TOF MS (Bruker ultraflXtreme) analysis. The remaining ~9 µL extended template-loop-primer solutions were used to test exonuclease activity.

**Comparison of SARS-CoV-2 exonuclease reaction for Tenofovir-terminated RNA with natural RNA.** The A-terminated RNA (2 µM) and Tenofovir extended RNA (2 µM) were annealed in Supplementary Table S-1 and were denatured at 95°C for 5 min, before being added to DMSO with 100 µg/ml 10% DMSO. Desalting was performed with an Oligo Clean & Concentrator kit (Zymo Research) resulting in ~10 µL purified aqueous RNA solutions. In all, 5 µL of a solution containing 80 µM GTP and 80 µM UTP in 1× reaction buffer was subjected to MALDI-TOF MS (Bruker ultraflXtreme) analysis.

**Yield-reduction assay.** Calu-3 cells (5 × 10⁴ cells/well) in 48-well plates were infected at an MOI of 0.1 for 1 h at 37°C. The cells were washed, and various concentrations of compounds were added to DMSO with 100 µg/ml 10% DMSO. Drug-screening assays were performed using SynergyFinder 2.0 software with the HSA method, in which the percentage of inhibition was plotted for curves of drugs alone and in combination.
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Author contributions
X.W., C.Q.S., S.J., O.A.C., C.T., N. F.-R., M.C., J.R.T., W.X., C.M., and A.G. performed the experiments. X.W., C.Q.S., S.J., C.T., N.F.-R., M.C., J.R.T., P.T.B., X.L., S.K., J.J.R., T.M.L.S., and J.J. analyzed the data. X.W., C.Q.S., S.J., C.T., M.C., J.R.T., X.L., S.K., P.T.B., J.J.R., T.M.L.S., and J.J. prepared the manuscript. C.Q.S., D.J.P., T.T., T.M.L.S., and J.J. conceptualized the experiments.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Thiago Moreno L. Souza or Jingyue Ju.

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