A dual mechanism of action of AT-527 against SARS-CoV-2 polymerase

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The guanosine analog AT-527 represents a promising candidate against Severe Acute Respiratory Syndrome coronavirus type 2 (SARS-CoV-2). AT-527 recently entered phase III clinical trials for the treatment of COVID-19. Once in cells, AT-527 is converted into its triphosphate form, AT-9010, that presumably targets the viral RNA-dependent RNA polymerase (RdRp, nsp12), for incorporation into viral RNA. Here we report a 2.98 Å cryo-EM structure of the SARS-CoV-2 nsp12-nsp7-nsp82-RNA complex, showing AT-9010 bound at three sites of nsp12. In the RdRp active-site, one AT-9010 is incorporated at the 3′ end of the RNA product strand. Its modified ribose group (2′-fluoro, 2′-methyl) prevents correct alignment of the incoming NTP, in this case a second AT-9010, causing immediate termination of RNA synthesis. The third AT-9010 is bound to the N-terminal domain of nsp12 - known as the NiRAN. In contrast to native NTPs, AT-9010 is in a flipped orientation in the active-site, with its guanine base unexpectedly occupying a previously unnoticed cavity. AT-9010 outcompetes all native nucleotides for NiRAN binding, inhibiting its nucleotidytransferase activity. The dual mechanism of action of AT-527 at both RdRp and NiRAN active sites represents a promising research avenue against COVID-19.
The highly conserved replicative enzymes of positive-sense RNA viruses, including the viral RdRp, remain at the forefront of drug-design strategies. Nucleoside analogs (NA) represent a promising class of RdRp inhibitors, and are currently used for the treatment of several other viral infections. NA prodrugs are metabolized in the host cell into active 5′-triphosphate forms that compete with natural nucleoside triphosphates (NTP) for incorporation into the viral RNA. This results in either chain-termination of viral RNA synthesis, or increases the viral mutation load to levels that can lethally alter the genetic make-up of the virus. Many details of NTP incorporation into viral RNA have been determined at the structural level following the pioneering work on the Reovirus RdRp1 (reviewed in ref. 2). However, CoVs stand out among RNA viruses for possessing an RNA-repair 3′-to-5′ exonuclease (ExoN, nsp14 stimulated by nsp10) able to excise mismatched bases as well as NAs incorporated into viral RNA, generally compromising the efficacy of these drugs3–6. Recently, the guanosine analog phosphoramidate prodrug AT-527 (Fig. 1a, left), was shown to act as a potent broad-spectrum anti-CoV inhibitor in a variety of cell lines7. It is now in phase III and II clinical trials for the treatment of COVID-19 and hepatitis C virus (HCV) infections8, respectively9 (www.clinicaltrials.gov/ct2). AT-527 and its active 5′-triphasphate AT-9010 (Fig. 1a, right) carries a 2′-fluoro-2′-C-methyl modified ribose, identical to that of the clinically relevant anti-HCV uracil prodrug Sofosbuvir10.

The CoV genome contains ~30,000 nucleotides, about three times more than that of other significant human pathogenic +RNA viruses (see ref. 11 for review). Genome size reflects the presence of novel domains, many of which remain poorly characterized. Among these is the Nidovirus RdRp-Associated Nucleotidyldtransferase (NiRAN) N-terminal domain of nsp12, just upstream of the RdRp12. The NiRAN is structurally related to the pseudo-kinase family of enzymes13, and has been shown to mediate the covalent transfer of nucleoside monophosphates (NMP) to viral cofactor proteins nsp7, nsp8 and nsp9, in a process known as NMPylation14–16. However, the role of this activity in the viral life-cycle remains unknown. The NiRAN has
additionally been proposed to participate in the guanylyltransferase step of viral RNA capping17. Mutations in the NiRAN are lethal to the virus12, making it an attractive candidate for CoV-specific drug targeting.

Here, we show that AT-9010 inhibits two separate enzyme activities of the SARS-CoV-2 replicase/transcriptase complex. First, it acts as an immediate RNA chain terminator at the RdRp active site and provides partial resistance to excision by the nsp14/nsp10 ExoN. Second, it is a NMPylation inhibitor of nsp8 and nsp9 when bound into a deep pocket located at the NiRAN active site.

Results

Structural studies of AT-9010 bound to the SARS-CoV-2 replicase:RNA complex. To investigate the AT-527/AT-9010 mechanism of action we performed cryo-EM studies with the SARS-CoV-2 minimal replication-transcription complex (RTC), comprised of nsp12 and essential cofactors nsp7 and nsp818,19. The complex was formed in the presence of both AT-9010 and an annealed primer-template RNA. Image processing of single particles allowed the reconstruction of RTC:RNA:AT-9010 complexes at 2.98 Å resolution (Supplementary Table 1 and Supplementary Fig. 1).

The overall RTC conformation resembles previous structures, with one nsp12, one nsp7 and two nsp8 proteins (Fig. 1b)20-22. Both nsp12 (residues 4-929) and nsp7 (residues 2-73) are almost fully resolved, while the two nsp8s are mostly resolved (residues 38-191 and 43-192). As observed in ref. 23, the dsRNA stabilizes both nsp8 alpha-helical extensions in their N-terminus regions. The RdRp domain adopts a canonical right-hand fold, with fingers, palm and thumb subdomains (for review24). As for all viral RdRps, the active site is formed by five conserved motifs, A-E, located on the palm subdomain, with two additional motifs, F and G, located in the finger subdomains. The fully resolved N-terminus NiRAN is located alongside the palm domain of the RdRp25,24 (Fig. 1b).

AT-9010 terminates the growing RNA chain. The 5' ′-end of the RNA template contains four consecutive cytidine bases (C24-C27), directing incorporation of AT-9010 into RNA (Figs. 1c, d and 2a). The 3′ ′-end of the RNA primer, is stabilized by Ser814 bonding through a non-bridging oxygen of the last phosphodiester bond. AT-9010 is incorporated at the first position (+1), base pairing with C27 of the template strand (Fig. 1b, c and Supplementary Movie 1). A second, free AT-9010 occupies the NTP-binding site, (−1) position, but is not correctly poised for incorporation (Fig. 1c). Palm domain residues Asp618 and Asp760 in motifs A and C, respectively, coordinate a single magnesium ion, which interacts with oxygens of the α and β-phosphates of the second AT-9010 (Fig. 2). Motif A and C acidic residues usually coordinate two metal ions25,26. The missing catalytic Mg2+ in the structure normally plays a critical role in the positioning of the 3′ ′-end of the RNA primer and the incoming NTP.

Chain-terminated RNA and next AT-9010 disrupts the catalytic site. The second, incoming (−1) AT-9010 guanine base is partially base-paired with cytosine (C26) of the template strand, and further stabilized by motif F Lys545 and motif B Ser682, two residues important during the fidelity check prior to incorporation (Figs. 1c and 2b-d)27,28. The α- and β- phosphates are coordinated by the single Mg2+, while the γ-phosphate is stabilized by motif A Lys621 and motif D Lys795 (Fig. 2d). The 3′ ′-OH of the elongated primer is too far from the α-phosphate for incorporation (6.3 Å) and furthermore the metal is not correctly aligned for catalysis. Rather, we observe that AT-9010 has its α-and β-phosphates spatially overlapping the expected position of the leaving pyrophosphate (β- and γ-phosphates) (Fig. 2c), as judged by comparing with either superimposed enterovirus RdRp structures or incorporated Remdesivir by SARS-CoV-2 nsp12,23,24.

The effect of the 2′-fluoro and 2′-C-methyl ribose modifications of the incorporated AT-9010 are as follows: The replacement of the 2′ hydroxyl by a fluoro group eliminates a stabilizing interaction with Ser759 of the SDD palm domain motif C. Comparison of the SARS-CoV-2 nsp12:RNA:Remdesivir and Favipiravir-ribose 5′-triphosphate cryo-EM structures with HCV NS5:RNA:Nucleotide ternary complexes26,28,29 shows a tight superimposition of catalytic residues, RNA backbone, and nucleotides/NAs except in the case of AT-9010 (Supplementary Fig. 2). The incoming AT-9010 ribose group is shifted in comparison to its expected position22,28,30 (Fig. 2c, d and Supplementary Fig. 2a, b, c). Its orientation is also different to that of pre-incorporated, (−1) translocated Sofosbuvir 5′-diphosphate and GS-9813 5′-diphosphate in HCV-RdRp structures29. As these two NAs carry the same 2′-fluoro-2′-C-methyl ribose as AT-9010, this demonstrates that this shift is neither dependent on the nature of the base nor on the ribose modification of the incoming NA 5′-triphosphate (Supplementary Fig. 2). Rather, the hydrophobic 2′-methyl group of the incorporated AT-9010 ribose creates a repulsive hydrophobic-polar contact with the ribose ring oxygen of the incoming NA (or NTP) (Fig. 2b), causing chain-termination. Following 2′-fluoro-2′-C-methyl NA incorporation into RNA by any given viral RdRp, this ribose modification may well promote a universal mechanism of chain-termination.

AT-9010 is a potent RNA chain terminator substrate. We compared AT-9010 incorporation and elongation by the purified, recombinant SARS-CoV and SARS-CoV-2 RTCs using a heteropolymeric RNA primer:template pair, corresponding to the 3′ end of the SARS-CoV-2 genome26. In the absence of GTP, AT-9010 is rapidly incorporated into viral RNA causing immediate termination of RNA synthesis (Fig. 3a). Even at concentrations of 500 µM of ATP (the next templated nucleotide), elongation past the incorporated AT-9010 is not observed (Supplementary Fig. 3a). Consistent with structural analysis, this indicates that chain-termination is independent of the identity of the incoming NTP. Rather, the AT-9010 ribose modification causes a misalignment that prevents further elongation (Fig. 2). Importantly, in the presence of equimolar concentrations of GTP, AT-9010 acts as a competitive guanosine substrate, discriminated against only ~5-fold (Fig. 3b). We additionally compared AT-9010 incorporation with the structurally related, uracil analog Sofosbuvir triphosphate (STP). Although STP is able to be incorporated by nsp12 as a substitute for UTP, it is not competitive with UTP, even at 5-fold higher concentrations (Supplementary Fig. 3b).

In both human bronchial and nasal epithelial cells incubated with 10 µM of the AT-527 prodruk, intracellular concentrations of AT-9010 peak at ~700 µM and 240 µM, respectively2. Considering an average intracellular concentrations of GTP of ~500 µM31, it is expected that AT-9010 would be highly competitive for insertion into viral RNA, frequently terminating synthesis.

RNA chain-terminated by AT-9010 shows resistance to ExoN removal. The stalling of the polymerase following insertion of a chain-terminating NA may allow excision by the proofreading 3′-to-5′ exonuclease nsp14/nsp106 potentially dampening AT-9010 efficacy. Following incorporation into RNA, both AT-9010 and STP are excised by the SARS-CoV-2 ExoN (Fig. 3b, c). Interestingly, however, AT-9010 is ~4.3-fold more resistant to excision.
relative to an unmodified or STP-terminated RNA 3′-end (Fig. 3b, c). Given the highly competitive incorporation of AT-9010, this reduced ExoN-rate likely slows viral replication, even if complete excision may eventually be achieved.

AT-9010 binds into the active site of the NiRAN domain. A third AT-9010 is found in the N-terminal NiRAN domain of nsp12 (Figs. 1b and 4 and Supplementary Movie 2). Initial attempts to place the AT-9010 triphosphate group lead to a steric clash between a non-bridging oxygen of the γ-phosphate and the carboxyl group of Asp218. However, the density suggests the presence of an additional metal ion, as seen in the SelO pseudo-kinase structure13. Density and coordination distances between this ion, the AT-9010-β-phosphate and the Asp218 carboxyl group are coherent, showing AT-9010 is in its diphosphate (DP) form—herein referred to as AT-9010-DP (Fig. 4b and Supplementary Fig. 4a). In support of the DP form, HPLC analysis of AT-9010 before and after incubation with either nsp12 wild-type (WT), or NiRAN mutant, or RdRp active-site mutant shows that the NiRAN domain mediates hydrolysis of the γ-phosphate. The presence of the DP form increases 2.3–2.8-fold with WT and RdRp active-site mutants, but not following incubation with the NiRAN active-site mutant (Supplementary Fig. 4b).

The NiRAN catalytic center contains a groove harboring two catalytic ions, coordinated by conserved residues Asn209 and Asp218 (Fig. 4c, e), leading to a narrow cavity. The α and β-phosphates of AT-9010 are stabilized in the groove by the two ions and residues Lys50, Lys73 and Arg116 (Fig. 4a). Intriguingly, the orientation of AT-9010-DP is strikingly different to that of ADP and GDP in existing NiRAN structures17,32, as well as other nucleotide-bound pseudo-kinase structures (Fig. S4a–c)20,21. Rather, the binding mode is reminiscent of the orientation of ATP bound to casein kinase33. The AT-9010-DP guanine base and modified ribose are accommodated in an inner narrow cavity (Fig. 4), which is unoccupied in existing NDP-NiRAN structures17,32. The γ-phosphates are coordinated in the same groove as the phosphates of GDP and ADP in other NiRAN structures, but are in a flipped orientation, occupying the usual positions of the β- and α-phosphates, respectively17,32. AT-9010-DP has thus a unique binding mode, driven by both the hydrophobic nature of the cavity and modified ribose.

AT-9010 inhibits nsp9 and nsp8 UMPylation. The NiRAN has previously been shown to mediate the covalent transfer of NMPs13 to various cofactor proteins14–16. In these studies, UTP
was demonstrated to be the preferred substrate for cofactor labeling. However, our structural analysis shows that the AT-9010 guanosine base is well accommodated into the NiRAN. To determine whether AT-9010 is able to inhibit NiRAN-mediated transferase activity, we performed competition experiments measuring the efficiency of nsp9-UMPylation by both SARS-CoV and SARS-CoV-2 RTCs in the presence of increasing concentrations of AT-9010, or its uracil equivalent STP (Supplementary Fig. 6a). Both drugs inhibit nsp9 labeling at comparable levels for SARS-CoV and SARS-CoV-2 RTCs. When provided at equimolar concentrations to UTP, nsp9-UMPylation is inhibited ~85–90% by both STP and AT-9010 for the SARS-CoV-2 complex, showing both drugs outcompete UTP for NiRAN-binding. Nsp8 has additionally been shown to act as a target substrate for NiRAN-mediated UMPylation by the CoV RTC7,16. Interestingly, AT-9010 is ~4-5-fold more efficient at blocking nsp8 labeling than the uracil equivalent STP (Supplementary Fig. 6b).

Furthermore, given the excess of nsp8 provided in the reaction, these inhibition data suggest that AT-9010 remains stably bound into the NiRAN active site, rather than being transferred to nsp8.

AT-9010 is stably bound in the NiRAN active site. Thermal shift assays with nsp12 confirm that AT-9010 provides more thermodynamic stability than any other native nucleotide (Supplementary Fig. 6c). Comparison of NiRAN and RdRp active-site mutants (K73A and SAA, respectively) shows that this stability increase is provided by AT-9010 binding preferentially to the NiRAN, rather than the RdRp active site. Both GTP: and AT-9010:nsp12 complexes show an increased thermal stability compared with UTP or STP-bound complexes. Overall, and consistent with structural analysis, these results indicate that guanosine is a stable ligand of the NiRAN active site, with the 2′-fluoro-2′-C-methyl ribose modification of AT-9010 likely allowing insertion into the pocket and/or providing additional stability.
In contrast, the binding of UTP is weak and putatively transient, likely to facilitate transfer to CoV nsps and possibly other targets.

**Discussion**

AT-9010 is efficiently incorporated into viral RNA, causes immediate chain-termination, and shows resistance to excision. Additionally, the superior affinity of guanosine analogs for the NiRAN as well as its deep AT-9010-binding pocket uncovers a specific druggable site of remarkable interest for anti-coronavirus research. The respective inhibition impact of AT-9010 on each of these activities is unknown. We note that the precise role of the NiRAN in the viral life-cycle is still somewhat speculative, but mutagenesis has shown that its NMPylation activity is essential.12

Nucleotide transferase enzymes are required for a range of reactions including, e.g., RNA capping, DNA/RNA ligation, and priming of RNA synthesis, making it possible that the NiRAN is involved in various viral processes. Currently, there is some evidence for both guanylyltransferase and RNA-priming activity for the NiRAN domain of SARS-CoV-2.16,17 Here, we show through both structural and biochemical analysis that AT-9010 binds preferentially, and stably in the NiRAN active-site pocket. Both of the reported NMPylation events (nsp8 and nsp9) are blocked efficiently by AT-9010. Therefore, any downstream activities dependent on this nucleotidylation event, or dependent on NiRAN-NTP binding in general (including capping, DNA/RNA ligation, or priming of RNA synthesis) would be blocked.

We note that the serendipitous simultaneous targeting of RdRp and NiRAN essential activities by the same drug adds momentum to the use of inhibitors against COVID-19 and the SARS-CoV-2 RTC, which remarkably has remained genetically stable since the beginning of the pandemic. Such AT-9010 pleiotropic action should attenuate the chance of simultaneous resistance mutations, and be an important asset in the control of the expanding genetic pool of SARS-CoV-2 variants observed in the current pandemic.

**Methods**

**5′-triphosphate nucleosides.** AT-9010, and 5′-triphosphate 2′-fluoro-2′-C-methyl uridine (Sofosbuvir 5′-TP, STP) were provided by NuBlocks LLC, Ontario, CA, USA. Other NTPs were HPLC grade, purchased from GE Healthcare.

**Expression and purification of SARS-CoV proteins.** The SARS-CoV and SARS-CoV-2 sequences used in this study are from the Tor2 (NCBI ref. seq.: NC_004718.3) and Wuhan-Hu-1 (NCBI ref. seq.: YP_009724389.1) isolates, respectively. Unless specified otherwise, a N-terminus 6His tag was fused to a TEV cleavage site ENLYFQG on which the last glycine was replaced by the first amino acid of the coding sequence of the gene of interest.

SARS-CoV proteins 6His(TEV)nsp7 and 6His(TEV)nsp8 were expressed under the control of a T5-promoter in pQE30 vectors in Escherichia coli (E. coli) NEB Express C2523 cells (New England Biolabs) carrying the pRare2LacI (Novagen) plasmid. Protein was expressed overnight at 17 °C (with 100 µg/mL ampicillin and 250 µM IPTG and 5 ng/mL of tetracycline for induction of chaperones proteins (groES-groEL-tig), and left overnight at 23 °C at 220 rpm. Cells were lysed over 0.25 mg/mL Lysozyme, 10 mM PMSF) and protein was removed via overnight cleavage with TEV protease (1:10 w/w ratio to TEV:protein) was performed prior to elution with 200 mM imidazole. The affinity tag was removed via overnight cleavage with TEV protease (1:10 w/w ratio to TEV-protein) in a dialysis buffer containing no imidazole and supplemented with 1 mM DTT. Cleaved protein was re-purified through a second cobalt column to remove the histidine-labeled TEV protease, and further purified with size exclusion chromatography (Cytiva Superdex S200) in a final buffer of 25 mM HEPES pH 8, 130 mM NaCl, 5 mM MgCl₂, and 5 mM TCEP.

SARS-CoV nsp12-8His was expressed from a pJ404 vector in E. coli strain BL21(pG/T2) (Takara 9124), in the presence of ampicillin (100 µg/mL) and Chloramphenicol (17 µg/mL). Expression was induced at OD₆₀₀ = 0.5–0.6 with 280 µM IPTG and 5 ng/mL of tetracycline for induction of chaperones proteins (groES-groEL-tig), and left overnight at 23 °C at 220 rpm. Cells were lysed over 45–60 mins at 4 °C, in a buffer containing 50 mM Tris pH 8, 300 mM NaCl, 5 mM
MgSO₄, 10% glycerol, 1% CHAPS, supplemented with 5 mM 2-mercaptoethanol, 0.5 mg/mL Lysozyme, 10 µg/mL DNase, 1 mM PMSF, 0.25 mg/mL Lysozyme, 10 µg/mL DNase, 1 mM PMSF, 0.2 mM benzamidine. NaCl was gradually increased to an optimal concentration of 300 mM to aid in the removal of contaminating nucleic acids. Following centrifugation (30,000 × g for 30 min), the supernatant was diluted to reduce NaCl concentration to 300 mM. Protein was purified using cobalt-based IMAC resin TALON® Superflow™ (Cytiva), washing three times with wash buffer (300 mM Tris pH 8, 10% glycerol) with alternating (Cytiva Superdex S200) in the same chromatography with HisPur Cobalt resin (Thermo Scientific). The cells were harvested then resuspended in buffer D (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM MgSO₄, 1 mM of β-mercaptoethanol, 0.25 mg/mL Lysozyme, 10 µg/mL DNase, 0.1% triton and 1 mM PMSF) and lysed by sonication. Protein was purified first through affinity chromatography with HisPur Cobalt resin (Thermo Scientific), eluted in 100 mM imidazole, then through size exclusion chromatography (GE Superdex S200) in a final buffer of 50 mM HEPES pH 7.5, 300 mM NaCl, 5 mM MgCl₂ and 1 mM of β-mercaptoethanol.

SARS-CoV protein nsp9 carrying a non-cleavable 6His tag in N-terminus was expressed under the control of a Tet-promoter in a pASK vector in E. coli NEB Express C2523 cells carrying the pCGL plasmid (New England Biolabs). Protein was expressed overnight at 20 °C with 50 µg/mL kanamycin, 17 µg/mL chloramphenicol), following induction with 200 µg/mL anhydrotetracycline at an O₆₀₀ = 0.6–0.7. Cells were incubated in lysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole, 5 mM MgSO₄, 1 mM of β-mercaptoethanol, 0.25 mg/mL Lysozyme, 10 µg/mL DNase, 0.1% Triton X-100 and 1 mM PMSF) and lysed by sonication. Protein was purified first through affinity chromatography with HisPur Cobalt resin (Thermo Scientific), eluted in 100 mM imidazole, then through size exclusion chromatography (GE Superdex S200) in a final buffer of 50 mM HEPES pH 7.5, 300 mM NaCl, 5 mM MgCl₂ and 1 mM of β-mercaptoethanol.

SARS-CoV-nsp9 was expressed with self-cleavable ubiquitin fused at its N-terminus and 6His at its C-terminus under the control of a Tet-promoter in a pASK vector in E. coli NEB Express C2523 cells carrying the pCGL plasmid (New England Biolabs). Protein was expressed overnight at 20 °C with 50 µg/mL kanamycin, 17 µg/mL chloramphenicol), following induction with 200 µg/mL anhydrotetracycline at an O₆₀₀ = 0.6–0.7. Cells were incubated in lysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole, 5 mM MgSO₄, 1 mM of β-mercaptoethanol, 0.25 mg/mL Lysozyme, 10 µg/mL DNase, 0.1% Triton X-100 and 1 mM PMSF) and lysed by sonication. Protein was purified first through affinity chromatography with HisPur Cobalt resin (Thermo Scientific), eluted in 100 mM imidazole, then through size exclusion chromatography (GE Superdex S200) in a final buffer of 50 mM HEPES pH 7.5, 300 mM NaCl, 5 mM MgCl₂ and 1 mM of β-mercaptoethanol.
heterogeneous refinement. A subset of 181,669 particles from the class with good features was subjected to Homogenous Refinement, Local Refinement and Non-uniform Refinement, resulting in a 2.98 A map.

Model building and refinement. To build the model of nsp12-nsp7-nsp8-RNA complex, the structure of SARS-CoV-2 nsp12-nsp7-nsp8-RNA complex (from PDB 7CYQ), with one nsp9 and nsp13 removed was placed and rigid-body fitted into the Cryo-EM map using UCSF Chimera. The model was manually built in Coor36 with the guidance of the Cryo-EM map, and in combination with real space refinement using Phenix.36 The model validation statistics are shown in Supplementary Table 1.

HPLC analysis of nsp12-AT-9010 products. Wild-type nsp12 of SARS-CoV and SARS-CoV-2, as well as SARS-CoV NiRAN (K73A) and RdRp active-site mutants (SDD — SAA) were incubated with equimolar concentrations of AT-9010 triphosphate for one hour at 37 °C in a reaction buffer containing 50 mM HEPES pH 7.5, 1 mM DTT and 1 mM MnCl. Reactions were heat inactivated at 70 °C for 10 min to promote dissociation of bound AT-9010 and products were purified through microcon spin columns (molecular weight cutoff 10,000). In parallel, a mock reaction without enzyme was performed under analogous conditions. Products were separated on HPLC C18 columns under a water: acetonitrile gradient to separate tri-, di- and mono-phosphorylated versions of AT-9010, UV detected and quantitated by determining peak surface areas.

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Acknowledgements

We thank Dr. Manfu Wang, and Dr. Zenglin Yuan from Wuxi Biortus Biosciences Co. Ltd. for their help in data processing and refinement. We thank Minqi Gao from Wuxi Biortus Biociences Co. Ltd. for his help in enzyme activity assays, and Dr. Zheng Liu from the Centre for Electron Microscopy, CUHK, for his kind help in data collection. We thank Léa Lo Bello for technical assistance in protein purification. We are in debt to Pr. Olve Peersen for his constant support, data sharing, and valuable biochemical insight during the course of the project. We thank Stephen Harrison, Sylvie Doublé, Julien Lescar, Olve Peersen, and Juan Reguera for helpful suggestions and critical reading of the manuscript. This project has received funding from the Fondation pour la Recherche Médicale (Aide aux Equipes), SCORE project H2020 SC1-PHE-Coronavirus-2020, grant#101003627, ATEA Pharmaceuticals, the Innovative Medicines Initiative 2 Joint Undertaking (IU) under grant agreement No. 101005077 (IMI-CARE).

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Competing interests
S.G., A.M., K.L., and J.P.S. are employees of ATEA Pharmaceuticals, Inc. The other authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-28113-1.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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