Supplementary information to

Viral polymerase binding and broad-spectrum antiviral activity of molnupiravir against human seasonal coronaviruses

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Supplementary Materials and methods

3D protein structure modeling of HCoVs RdRp

A homology-based approach was applied to model the 3D protein structure of HCoVs RdRp. We used the MODELLER 9.24 for modeling and loop refinement of the structure. The full-length sequence of HCoVs RdRp was obtained from the NCBI protein database (Accession AIW52828.1) and protein-protein BLAST tool was used for finding the most similar protein structure in the PDB database. The structure of SARS-CoV-2 RdRp (PDB Id 7C2K) with highest similarity was retrieved from the PDB database, and its Chain A was used as a template for modeling. The MODELLER 9.24 program

was run using the query and template sequence alignment file in PIR format. The validation of the modelled structure was performed using PROCHECK and ERRAT programs. The outlier's residues in the Ramachandran plot were corrected through loop refinement in MODELLER 9.24. DOPE (discrete optimized potential energy) score was used to select the best model for further in silico stereochemical quality assessment and docking studies. Finally, GalaxyWeb was used for overall structure refinement of the modelled protein. The final model of protein structure was re-analyzed by Swiss-model structure assessment (generating the Ramachandran plot and QMEAN score), PROCHECK and ERRAT. Chimera tool were used to perform the superimposition for comparative analysis of target and template structure to calculate the root mean square deviation (RMSD) and to energetically minimize the structure for docking studies. BIOVIA Discovery studio visualizer was used for image preparation of modelled structures.

Protein-ligand docking

Molecular docking of SARS-CoV-2, HCoV-NL63, HCoV-OC43 and HCoV-229E RdRps and the polymerase-RNA complexes with the active form of molnupiravir NHC (PubChem CID: 145996610) were performed with the LibDock program in Discovery Studio Client v20.1.0.19295 (Discovery Studio, Accelrys Software Inc./BIOVIA, San Diego, CA). LibDock is a flexible docking module, which uses protein site features referred to as hot spots consisting of two states (polar and apolar). The ligand poses are placed into the polar and apolar receptor interaction sites. A polar hotspot is preferred by a polar ligand atom (e.g., a hydrogen bond donor or acceptor), and an apolar hotspot is preferred by an apolar atom (e.g., a carbon atom). The protocol allows the user to specify several modes for generating ligand conformations for docking. Conformer Algorithm based on Energy Screening and Recursive buildup (CAESAR) was used for generating the conformations. The smart minimizer was used for *in situ* ligand minimization. To identify interacting residues of the

receptor with the bound ligand, a 2D diagram of receptor-ligand interactions was constructed. Scoring was performed using various scoring functions including Jain, Ludi, the potential of mean force (PMF), and piecewise linear potential (PLP1) to evaluate ligand binding in the receptor cavity.

Reagents and antibodies

Molnupiravir (MedChem Express, USA) and GC376 (AOBIOUS) were dissolved in dimethyl sulfoxide (DMSO, Sigma, Zwijndrecht, The Netherlands). Anti-double-stranded-RNA (dsRNA) antibody (SCIONS J2 monoclonal antibody) was purchased from English&Scientific Consulting Kft. Anti-mouse IgG (H&l Alexa Fluor®594, Abcam) was used as secondary antibody.

Viruses and cell lines

Monkey LLCMK-2 cells were cultured in minimal essential medium with Earle's salt (MEM; Gibco, Grand Island, USA) containing 8% (vol/vol) heat-inactivated fetal calf serum (FCS, Sigma–Aldrich, St. Louis USA), 1% (vol/vol) nonessential amino acid (Sciencell, San Diego, California, USA), 0.1% (vol/vol) L-Glutamine (Lonza, Verviers, Belgium), 100 IU/mL Penicillin and 100 mg/mL Streptomycin (Gibco, Grand Island, USA). Multiple cell lines including human colon cancer cell line Caco2, human hepatoma cell line Huh7, monkey kidney cell line Vero-E6 and adenocarcinomic human alveolar basal epithelial cell line A549 were cultured with Dulbecco's modifified Eagle medium (DMEM) (Lonza Biowhittaker, Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Sigma–Aldrich, St. Louis USA), 100 IU/mL Penicillin and 100 mg/mL Streptomycin (Gibco, Grand Island, USA). HCoV-NL63 stock was produced by consecutively inoculating the virus onto LLCMK-2 cells. HCoV-OC43 and HCoV-229E were bought from ATCC (USA) and amplified in Huh7 cells. Cell lines were analyzed by genotyping and confirmed to be mycoplasma negative.

Virus production and inoculation re-infection assay

LLCMK-2 cells harboring the infectious HCoV-NL63 were seeded into multi-well plates, culturing at 33 °C, with 5% CO_2 for 5-7 days, over 50% of cells have cytopathic effect (CPE), then harvested HCoV-NL63 particles by repeated freezing and thawing three times, then filtered with 0.45 μm filters. Huh7 cells harboring the infectious HCoV-OC43 or HCoV-229E were seeded into multi-well plates, culturing at 33 °C, with 5% CO_2 for 4-6 days, over 50% of cells have cytopathic effect (CPE), HCoV-OC43 or HCoV-229E particles were harvested by repeated freezing and thawing three times, then filtered with 0.45 μm filters. Cells were seeded into multi-well plates and culture medium was discarded when cell confluence was approximately 80%, followed by twice washing with 1×PBS. Harvested HCoV-NL63 were added, incubated overnight and harvested HCoV-OC43 or HCoV-229E were added, incubated for 2 h at 33 °C, with 5% CO_2, followed by three times washing with 1×PBS to remove unattached viruses. Then cells were incubated with culture medium for another 48 h. The infectivity of produced HCoVs particles were analyzed by qRT-PCR and confocal imaging assays, respectively.

Antiviral drug treatment

LLCMK-2, Huh7 and Caco2 cells were first inoculated with HCoV-NL63 at a multiplicity of infection (MOI) of 0.1, and incubated at 33 °C overnight, A549 cells were first inoculated with HCoV-229E or HCoV-OC43 at a multiplicity of infection (MOI) of 0.1, and incubated at 33 °C 2 h. The cells were then washed twice with PBS to remove free virus particles and treated with molnupiravir for the indicated time period. Cells, total RNA or supernatant were collected for further analysis.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated using Macherey-Nagel NucleoSpin® RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000

(Wilmington, DE, USA). cDNA was synthesized by using a cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan). Real-time PCR reactions were performed with SYBR-Green-based real-time PCR (Applied Biosystems®, Austin, USA) on a StepOnePlusTM System (Thermo Fisher Scientific LifeSciences). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as housekeeping gene. Relative gene expression of target gene was normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$, $\Delta\Delta CT = \Delta CTsample - \Delta CTcontrol$ (ΔCT = CT [target gene] - CT[GAPDH]). Template control and reverse transcriptase control were included in all qRT-PCR experiments, and all primers are listed in Supplementary table 1.

Confocal fluorescence microscopy

Caco2 or A549 cells cultured in an 8-well chamber (cat. no. 80826; ibidi GmbH) were inoculated with HCoV-NL63, HCoV-229E, HCoV-OC43 respectively at 0.1 MOI, and incubated at 33 °C overnight or 2 h. The culture medium was then replaced by medium containing different concentrations of molnupiravir, and the cells were cultured for another 48 h. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeablized with 0.2% (vol/vol) Triton X-100 for 10 min, blocked with milk-tween-glycine medium (0.05% tween, 0.5% skim milk and 0.15% glycine) for 1 h, and reacted with anti-dsRNA antibody (1:200) diluted in blocking solution at 4 °C overnight. The cells were then incubated with 1:1000 dilutions of the anti-mouse IgG secondary antibodies for 1 h. Nuclei were stained with DAPI (4, 6-diamidino-2-phenylindole; Invitrogen). Images were detected using Leica SP5 cell imaging system.

MTT assay

LLCMK-2, Huh7, Caco2, A549 cells were seeded into 96-well tissue culture plates (1×10^4cells/well), and then treated with the indicated compounds for 48 h. Cells were incubated with 10 μL 5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)

-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 3 h, then replaced with 100 µL DMSO medium and incubated at 37°C for 30 minutes. The absorbance at 490 nm was recorded using a microplate absorbance reader (Bio-Rad, CA, USA).

Quantification of HCoVs genome copy numbers

An amplicon of HCoV-NL63, HCoV-229E or HCoV-OC43 (a fragment of N protein) were cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA) to generate a template for quantifying HCoVs genome copy numbers. The plasmid was extracted by Quick Plasmid Miniprep Kit (Invitrogen, Lohne, Germany). A series of dilutions (from 10^{-1} to 10^{-9}) were prepared and then were amplified and quantified by qRT-PCR to generate a standard curve. This standard curve was generated by plotting the log copy number versus the cycle threshold (CT) value (Fig. S5). Copy numbers were calculated by using the following equation: Copy number (molecules/µl) = [concentration (ng/µL) × 6.022× 10^{23} (molecules/mol)]/ [length of amplicon× 640 (g=/mol) × 10^{9} (ng/g)].

TCID50 assay

Viruses in the cultured cells the supernatant were harvested through repeated freezing and thawing for three times. HCoVs titer were quantified by using a 50% Tissue Culture Infectious Dose (TCID50) assay. Briefly, ten-fold dilutions of HCoV-NL63 were inoculated onto LLCMK-2 cells, HCoV-229E were inoculated onto Huh7 cells, HCoV-OC43 were inoculated onto Vero-E6 cells, grown in a 96-well tissue culture plate at 2,000 cells/well. The plate was incubated at 33 °C for 4-7 days, and each well was examined under a light microscope for cytopathic effect (CPE). The TCID50 value was calculated by using the Reed-Muench method.

Serial passaging of HCoV-NL63 with molnupiravir treatment

HCoV-NL63 were passaged in Caco2 cells in the absence of drug (vehicle control) or in the presence of gradually increasing concentrations of the drug (5

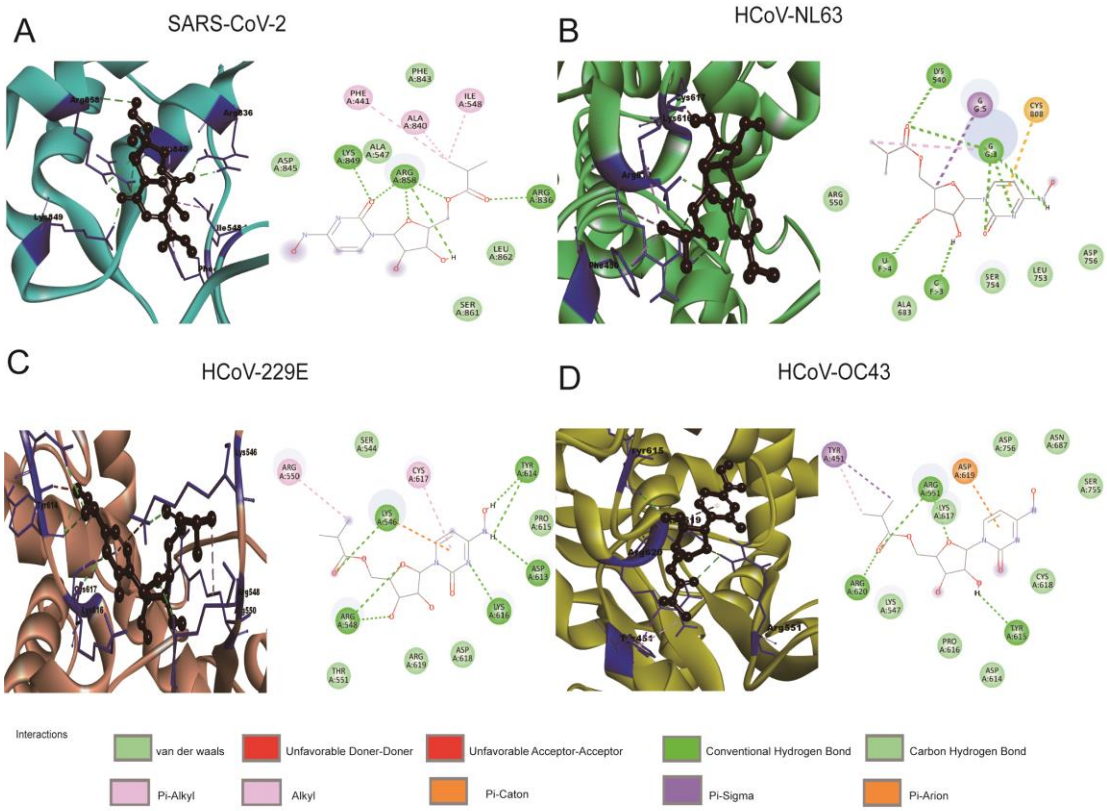
μM of molnupiravir for passage 1–10 and 10 μM of molnupiravir for passage 11–20). In brief, Caco2 cells in 6-well plate were inoculated with virus (MOI = 0.5) at 33 °C for overnight, followed by adding molnupiravir or without drug (as control). After 48 h, both cells and supernatant were harvested, subsequently frozen, thawed once, and centrifuged. The supernatant containing passaged viruses was stored at −80 °C until used for the next passage. Viruses were serially passaged by using 1 aliquot of viral stock from the preceding passage to infect fresh Caco2 cells. The effect of each passage of virus (same titer) was quantified by qRT-PCR.

Statistics analysis

All numerical results were reported as Mean ± SEM. The statistical significance of differences between means was assessed with the Mann-Whitney test (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA). The threshold for statistical significance was defined as $P \leq 0.05$.

Supplementary Table 1. Primers used in the study

Gene name	F-sequence (5' to 3')	R-sequence (5' to 3')
HCoV-NL63 plasmid	ACGCAATGCCACTGTTGTTA	GACAACACCGTCATCAGAGA
HCoV-229E plasmid	ATGGCTACAGTCAAATGGGC	AAGAAGCAGGACTCTGATTACG
HCoV-OC43 plasmid	GGGAAAGGAGTTTGAGTTTGTAG	CTCGTCAGGATTCCCAGATAA
Human GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGTAGCCA A
NL63	CTTCTGGTGACGCTAGTACAGCTTAT	AGACGTCGTTGTAGATCCCTAACAT
229E	GTCGTCAGGGTAGAATACCTTA	CCCGTTTGCGCTTTCTAGT
OC43	AGCAACCAGGCTGATGTCAATACC	AGCAGACCTTCCTGAGCCTTCAAT

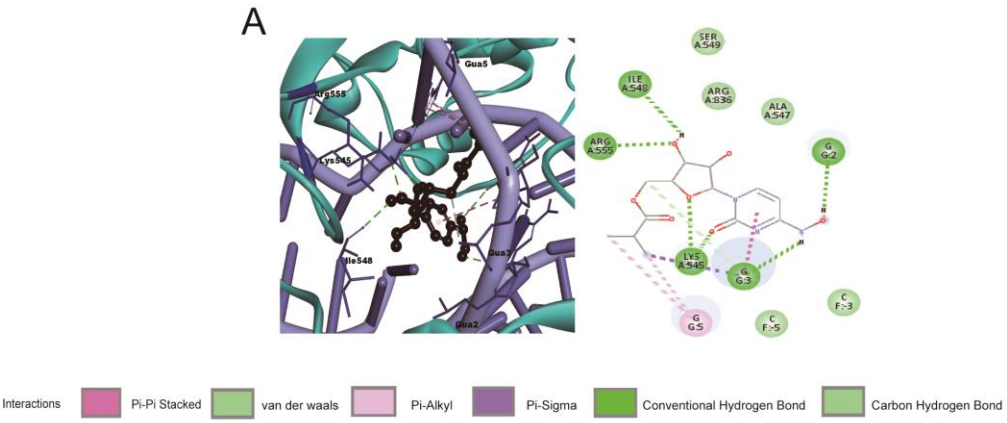


E

RdRp	Dock	Active Site Amino Acids	Interacting Amino acids	B.E.
SARS CoV2	Without RNA	545-555	547, 548	-6.1
NL63	Without RNA	540-550	548, 549, 550	-6.6
229E	Without RNA	540-550	544, 546, 548,550	-6.5
OC43	Without RNA	541-551	547, 551	-6.9

Supplementary Fig. S1. Site specific binding mode of NHC to coronavirus RdRp. The active form of molnupiravir (β-D-N4-hydroxycytidine triphosphate; NHC), binding to the RdRp (atom color ribbons) of SARS-CoV-2 (A), HCoV-NL63 (B), HCoV-229E (C) and HCoV-OC43 (D), is depicted as surface representation. H-bond donor (purple) and acceptor (green) interactions are depicted. (E) Summary of binding mode and affinity index (B.E.).

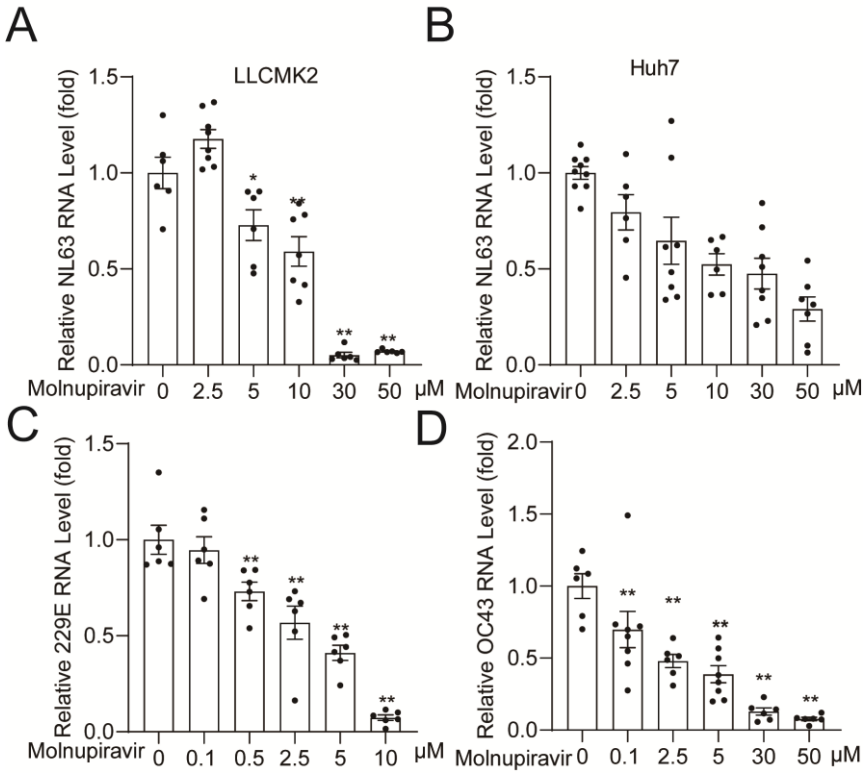
A



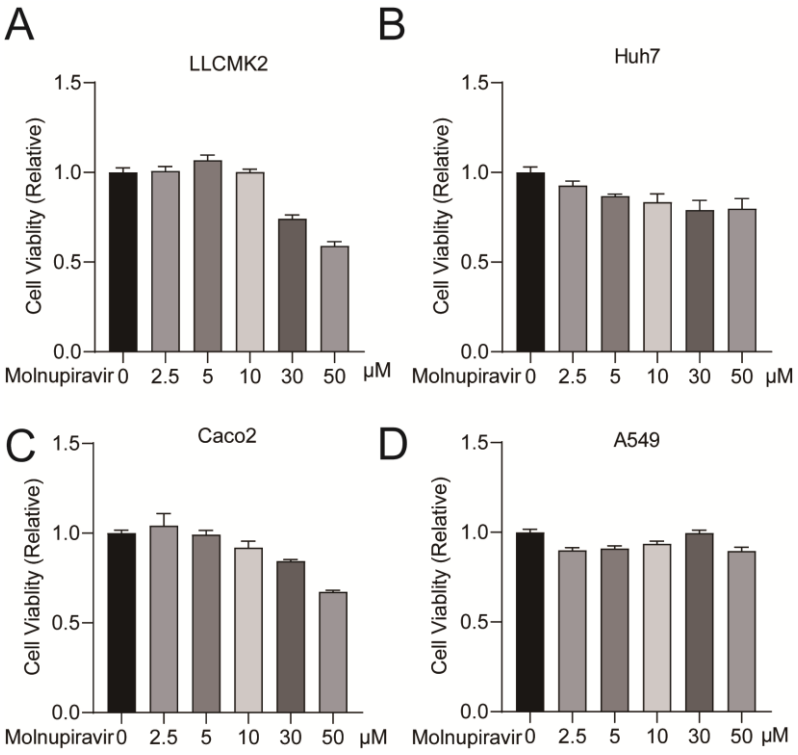
B

RdRp	Dock	Active Site Amino Acids	Interacting Amino acids	B.E.
SARS CoV2	With RNA	545-555	545, 547, 548, 549, 555	-8.0

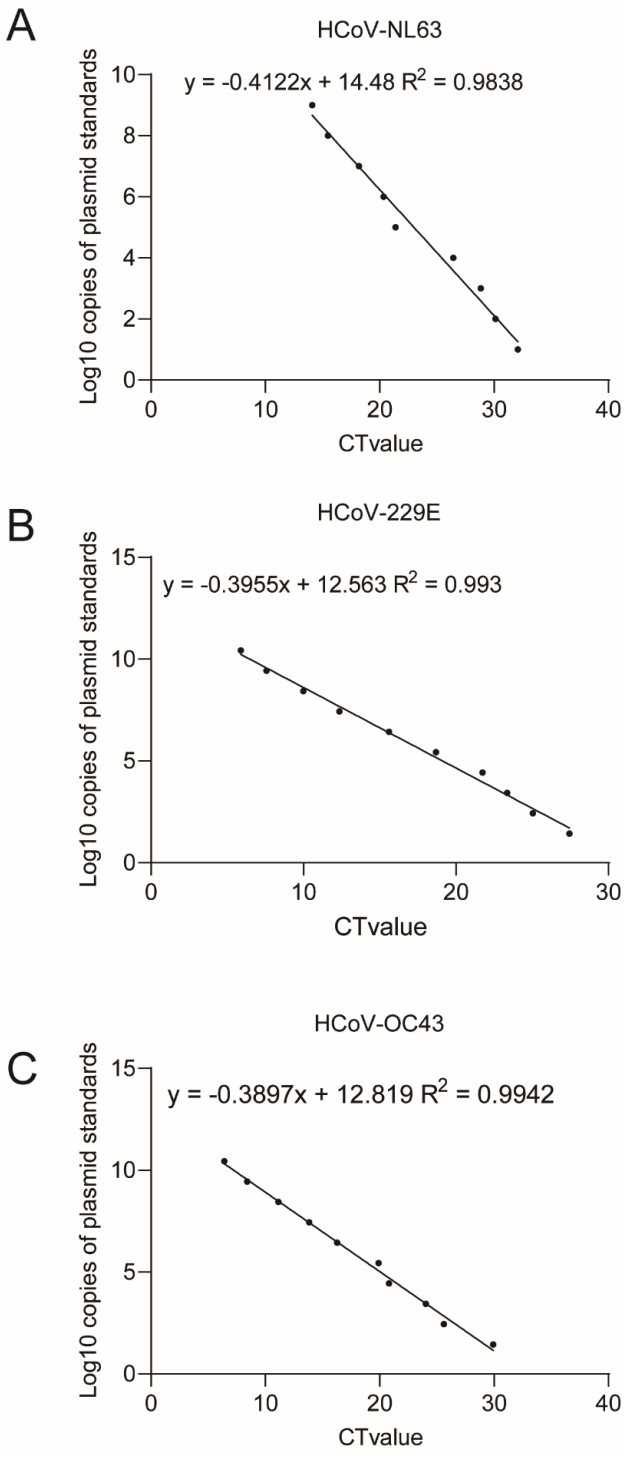
Supplementary Fig. S2. Site specific binding mode of NHC to SARS-CoV-2 polymerase-RNA complex (crystal structure). (A) The active form of molnupiravir (β-D-N4-hydroxycytidine triphosphate; NHC), binding to the RdRp -RNA complex (atom color ribbons) of SARS-CoV-2. (B) Summary of binding mode and affinity index (B.E.).



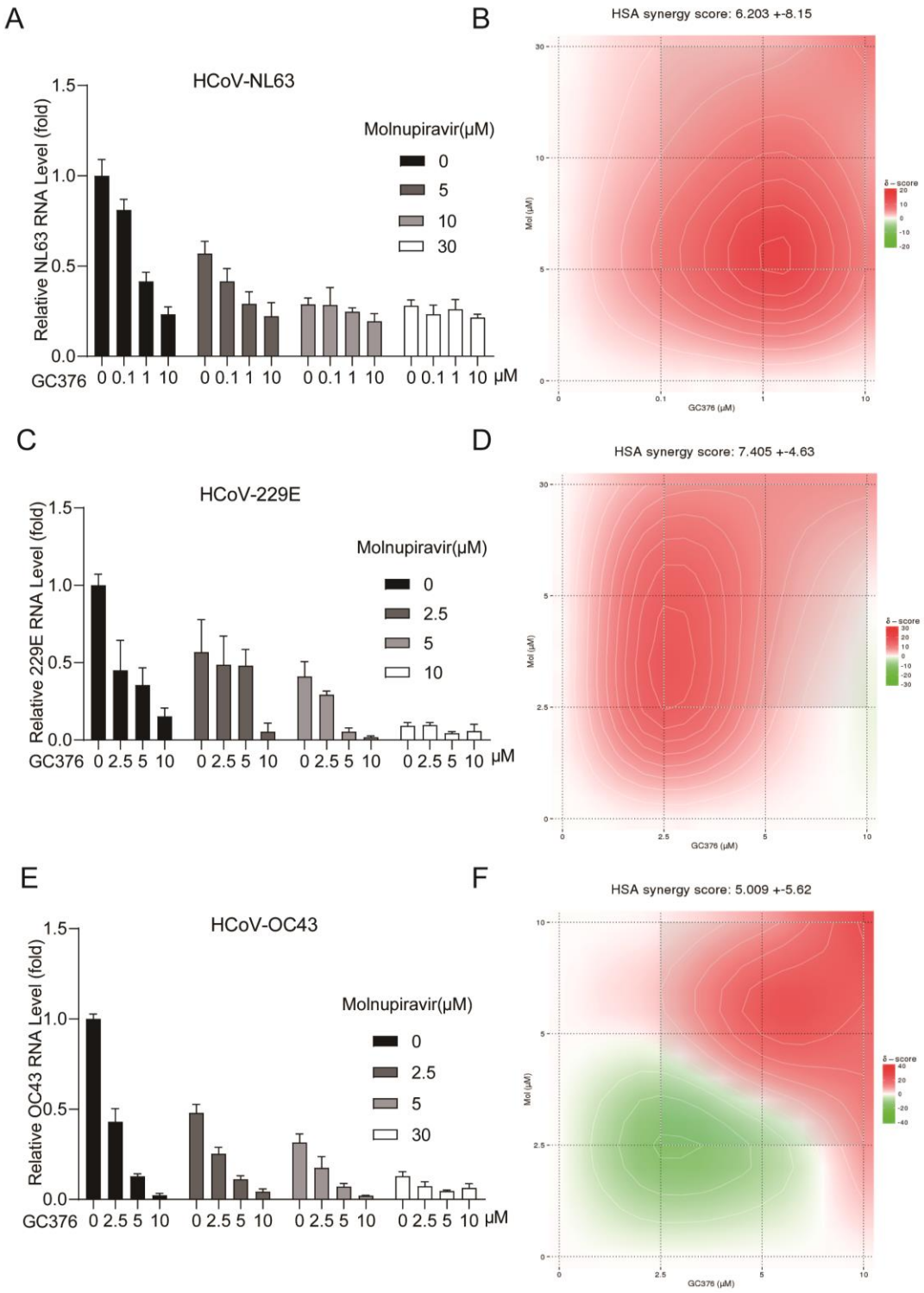
Supplementary Fig. S3. Antiviral effects of molnupiravir against HCoVs in different cell culture models. (A) and (B) Dose-dependent inhibition of HCoV-NL63 replication in LLCMK-2 and Huh7 cell lines by molnupiravir treatment. Intracellular viral RNA quantified by qRT-PCR was normalized to housekeeping gene GAPDH and presented relative to the control (CTR) (set as 1) (n = 6). (C) and (D) Dose-dependent inhibition of HCoV-229E or HCoV-OC43 replication in A549 cell line by molnupiravir treatment. Intracellular viral RNA quantified by qRT-PCR was normalized to housekeeping gene GAPDH and presented relative to the control (CTR) (set as 1) (n = 6-8).



Supplementary Fig. S4. The cytotoxicity of molnupiravir on different cell lines. (A), (B), (C) and (D) LLCMK-2, Huh7, Caco2 and A549 cells treated with different concentrations of molnupiravir for 48 hours. Cytotoxicity was determined by MTT assay (n = 8-16).

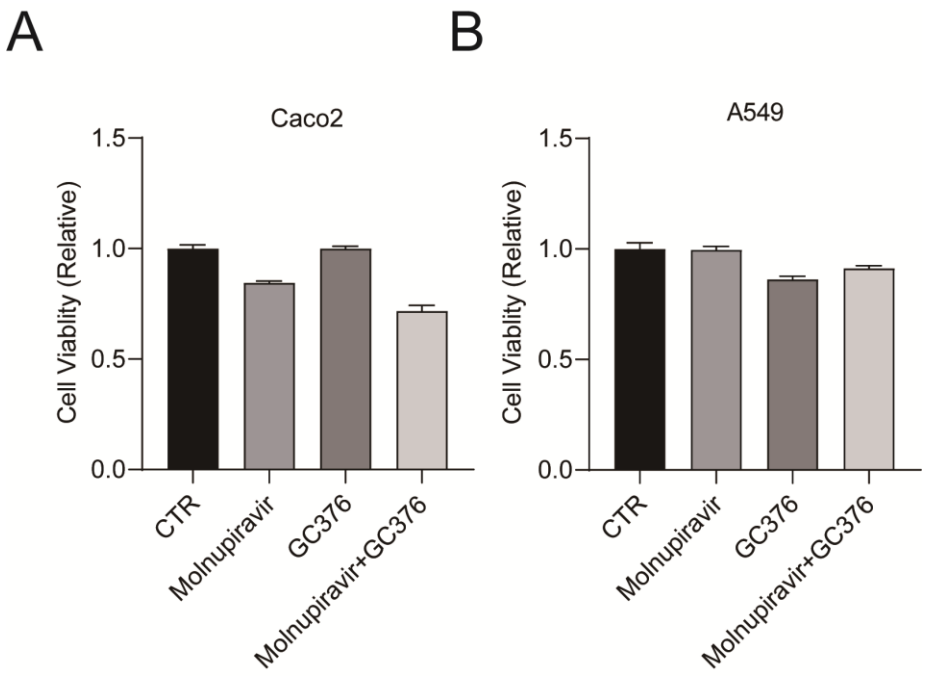


Supplementary Fig. S5. (A), (B) and (C) Standard curve for quantifying HCoVs genome copy numbers. Amplicon of the N protein of HCoVs were cloned into the pCR2.1-TOPO vector. The plasmid was extracted, followed by a series of dilutions from 10^{-1} to 10^{-9} and then were amplified and quantified by qRT-PCR. Standard curve was generated by plotting the cycle threshold (CT) value regarding the log copy numbers.



Supplementary Fig. S6. The effects of combining molnupiravir with GC376 on seasonal coronavirus infections. (A), (C) and (E) The antiviral effects of combining various concentrations of molnupiravir and GC376. Data represent as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. (B), (D) and (F) Synergy plot representing the score of antiviral activity for the molnupiravir-GC376

combination based on the data shown in A, C and E (n = 4-6).



Supplementary Fig. S7. (A) and (B) Cytotoxicity of molnupiravir, GC376 or their combinations. Caco2 or A549 cells were treated with different concentrations of molnupiravir (30 μM), GC376 (10 μM) or their combinations respectively for 48 h. Cytotoxicity was determined by MTT assay (n = 8-16).