Activity profiling of deubiquitinating inhibitors-bound to SARS-CoV-2 papain like protease with antiviral efficacy in murine infection model

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Abstract

Emerging variants of SARS-CoV-2 and drug-resistant mutants jeopardizes the efficacies of currently deployed therapies, highlighting the necessity for development of more efficacious antivirals. Given the highly conserved sequence and pivotal role of the papain-like protease (PLpro) in the viral replication and pathogenesis, it is a promising yet a challenging drug target. Additionally, PLpro plays a very important role in modulation of host immune response by deubiquitinating (DUBs) or deISGylating host proteins. Thus, targeting PLpro serves as a two-pronged approach to abate SARS-CoV-2. Owing to the structural and functional similarities of PLpro with the cellular DUB enzymes, this study developed an *in-house* library of DUB inhibitors and leveraged structure-based identification tools to identify five promising inhibitors targeting the Ubiquitin/ISG15 binding site and the known BL2 groove pocket of PLpro. The proteolytic activity of purified PLpro was markedly inhibited by four of these compounds and these displayed potent antiviral efficacies against SARS-CoV2 infection in a dose dependent manner. Crystal structure of SARS-CoV-2 PLpro in complex with two of these DUB inhibitors, the Linagliptin and Lithocholic acid revealed insights into the molecular inhibition mechanism with unique interactions within the Ubiquitin/ISG15 binding site (S2 site; Phe69, His73, Asn128, His175) and the substrate binding cleft. Additionally, oral and intraperitoneal treatments with Linagliptin increased survival, reduced lung viral load, and ameliorated histopathological damage in mouse-adapted model of SARS-CoV-2 infection. For the first time, this study demonstrated a two-pronged strategy using DUB inhibitors that target the PLpro and simultaneously reinstates the host's immune response against SARS-CoV-2.

Keywords: PLpro, SARS-CoV-2, COVID-19, antivirals, protease

Introduction

The pandemic of COVID-19, caused by the SARS-CoV-2 and the resurgence of drug-resistant variants, has taken a heavy toll on global health and calls for effective and safe anti-SARS-CoV-2 drugs. The rapid rollout of vaccine-based prophylactic therapies against SARS-CoV-2 initially saved numerous lives but the resulting immune pressures from vaccinations and infections led to emergence of highly contagious immune escape variants (Baker et al., 2021; Dubey et al., 2021; Mudgal et al., 2020). Concerns have been raised about the diminishing range, strength, and duration of vaccinal as well as natural immunity, which could compromise the geographical or temporal efficacy of these interventions and could lead to breakthrough infections (Baker et al., 2021; Garcia-Beltran et al., 2021).

Precedent studies on SARS-CoV-2 highlight that the catalytic site of certain non-structural proteins (nsP) of SARS-CoV-2, such as papain-like protease (PLpro), 3-chymotrypsin-like protease (3CL pro or Mpro), RNA-dependent RNA polymerase (RdRp), and helicase display high levels of sequence similarity with their corresponding SARS and MERS counterparts and represent promising druggable targets for therapeutic development (Abdelnabi et al., 2021; Driouich et al., 2021; Li and De Clercq, 2020; Rani et al., 2022, 2020; Shan et al., 2021). Several small molecule therapeutics or neutralizing antibodies, either clinically approved or in late-stage of development, are targeting the key viral proteins such as the Spike protein, nucleocapsid, Mpro and the RdRp (Choudhary et al., 2020; Cui et al., 2024; Dhaka et al., 2023; Krammer, 2020; Li et al., 2022; Singh et al., 2024). Examples of the FDA approved direct-acting antivirals include Remdesivir and Molnupiravir, which inhibits SARS-CoV-2 RdRp, and the Mpro inhibitor, Paxlovid (Kokic et al., 2021; Qiao et al., 2021; Wenhao et al., 2020; Zhang et al., 2020). Nevertheless, mutant SARS-CoV-2 strains with resistance against Remdesivir and Paxlovid have been detected in cell culture and drug treated COVID-19 patients, raising a demand for additional antivirals with alternative modes of action (Hirotsu et al., 2023; Hu et al., 2023; Iketani et al., 2023; Stevens et al., 2024). Indeed, PLpro presents to be a more challenging and potentially promising druggable target of SARS-CoV-2 because of the shallow S1 and S2 sites, and effective inhibitors are yet to be discovered for it (Tan et al., 2024).

The genome of SARS-CoV-2 comprises of a single-stranded positive-sense RNA of ~ 30kb, which is translated to produce structural, non-structural, and accessory viral proteins, essential for carrying out replication, maturation, and genome packaging of SARS-CoV-2 (Arya et al., 2021; V'kovski et al., 2021). The genomic RNA gets translated to produce two overlapping polyproteins, pp1a and pp1ab (formed after -1 ribosomal frameshifting mechanism), that are proteolytically cleaved and processed into 16 nsPs by two virus-encoded cysteine proteases, the PLpro and the Mpro (Osipiuk et al., 2021; Ren et al., 2021). These nsPs form the replication and transcription complex (RTC) responsible for directing the process of transcription, replication, and maturation of the viral genome (Arya et al., 2021; V'kovski et al., 2021). SARS-CoV-2 PLpro, a domain within nsP3 protein, is a cysteine protease that recognizes the P4-P1 consensus LXGG sequence and cleaves the peptide bond between nsP1/nsP2

(LNGG↓AYTR), nsP2/nsP3 (LKGG↓APTK), and nsP3/nsP4 (LKGG↓KIVN) (Shan et al., 2021; Wioletta et al., 2021). It shares a sequence identity of 83% with PLpro of SARS-CoV and is relatively distant from MERS with a sequence identity of 32.9% (Shin et al., 2020; Weglarz-Tomczak et al., 2021). As an evasion mechanism against the host innate immune response, PLpro possesses deubiquitination and deISG15ylation activities that cleave off ubiquitin (Ub) and ISG15 (ubiquitin-like interferon-stimulated gene 15) post-translational modifications from host proteins by recognizing the C-terminal RLRGG sequence (McClain and Vabret, 2020; Shen et al., 2022; Shin et al., 2020). Interestingly, the multifunctional PLpro inactivates TBK1, a kinase required to activate transcription factor interferon responsive factor 3 (IRF3), prevents dimerization and translocation of IRF3 to the nucleus, and blocks NF-κB signaling pathway, eventually attenuating the type I interferon response in the host cell (Fu et al., 2021; McClain and Vabret, 2020; Osipiuk et al., 2021). Due to the dual role of PLpro in replication and pathogenesis of SARS-CoV-2, targeting PLpro may have an advantage in not only suppressing viral infection but also in restoring the dysregulated signaling cascades in infected cells that may otherwise lead to cell death in the surrounding, non-infected cells. Despite extensive efforts in drug designing and lead optimization, the development of PLpro inhibitors is challenging and still remain at the preclinical stage with only a few covalent and non-covalent inhibitors reported till date (Choudhary et al., 2023; Fu et al., 2021; Kiira et al., 2008; Patchett et al., 2021).

Of note, PLpro has a right-handed "thumb-palm-finger" architecture which is similar to that of host deubiquitinating enzymes (DUBs), the ubiquitin-specific protease (USP), despite very low sequence similarity between them (~10%) (Osipiuk et al., 2021). SARS-CoV-2 PLpro has two domains, the "thumb-palm-finger" catalytic domain at the C-terminus and a small N-terminal ubiquitin-like (UBL) domain (Fu et al., 2021; Klemm et al., 2020; Osipiuk et al., 2021). The active site of PLpro is made up of a canonical cysteine protease triad of Cys111, His272, and Asp286 residues, located at the interface of thumb and palm sub-domains. The most complex architecture is of the fingers subdomain, as it is made up of two α-helices, six β-strands, and a zinc-binding loop that is indispensable for maintaining structural integrity (Gao et al., 2021; Klemm et al., 2020; Osipiuk et al., 2021). An important flexible β-turn/loop, blocking/binding loop 2 (BL2 loop) that recognizes the P2-P4 of the LXGG motif of the substrate and closes upon binding of substrate/inhibitor, is located between β11–12 strands spanning the residues from 267–271 (Gao et al., 2021; Klemm et al., 2020; Shin et al., 2020).

Interestingly, the PLpro shares a strikingly similar USP fold like the cellular DUBs, with a structurally conserved active site fold and orientation of catalytic triad residues Cys-His-Asp/Asn. Previous studies have reported successful identification and repurposing of USP inhibitors against SARS-CoV and MERS (Chou et al., 2008). Moreover, inhibitors with a cyanopyrrolidine warhead, such as gliptins, are reported to initiate a β-elimination reaction in the active site of USPs, resulting in irreversible inactivation of active site cysteine to dehydroalanine after desulfhydration reactions (Bashore et al., 2020; Jadav et al., 2012). These gliptins are already in clinical use as an antidiabetic compound inhibiting dipeptidyl peptidase 4 (DPP4), a

serine protease and a possible receptor for SARS-CoV-2 entry (Fisman and Tenenbaum, 2015; Jadav et al., 2012; Pitocco et al., 2020). Notably, the DPP4 inhibitor, sitagliptin, is reported to exert an anti-inflammatory effect on the lungs by inhibiting the IL-1 and IL-6 inflammatory pathways, eventually providing some relief against acute respiratory distress syndrome (ARDS) (Kawasaki et al., 2018; Valencia et al., 2020; Yang et al., 2021). Therefore, repurposing gliptins and DUB/USP inhibitors are hypothesized to serve a dual mode of action in inhibiting SARS-CoV-2; firstly, by the possible inhibition of enzymatic activity of PLpro due to structurally conserved active site fold and secondly, via restoration of host innate immune responses.

The present study aims to identify and repurpose the available DUB inhibitors and cyanopyrrolidine ring containing compounds against PLpro of SARS-CoV-2 by simultaneously engaging the catalytic site and the ISG15/Ub S2-binding pocket of PLpro, an approach which has not been previously exploited for identification of SARS-CoV-2 antivirals. High-throughput virtual screening, docking, and simulation experiments identified five compounds with docking scores comparable to known PLpro inhibitor, GRL0617, suggesting their potentiality as SARS-CoV-2 inhibitors. Two of these compounds, Lithocholic acid and Linagliptin exhibited significant binding affinity, and greater inhibitory potency against PLpro enzyme and SARS-CoV-2 viral replication inhibition across multiple cell lines. Next, to dissect the mechanism of action of identified inhibitors, the crystal structures of Linagliptin and Lithocholic acid in complex with PLpro were determined to expose inhibitory sites and to gain structural insights into the inhibitory mechanisms. Further, the compound, Linagliptin, was selected as an optimized lead and it's anti-SARS-CoV-2 activity was characterized *in vivo* using a mouse-adapted SARS-CoV-2 model. Herein, this study opens a new avenue for repurposing DUB inhibitors as dual-mechanistic antivirals against SARS-CoV-2.

Results

PLpro is a deubiquitinating and deISGylating enzyme

Structural studies have contributed to a detailed understanding of SARS-CoV-2 PLpro with other DUBs. Reminiscent of the overall architecture of DUBs of the USP family, PLpro shares a similar active site architecture with host USP7 (also known as HAUSP) and USP2 proteins, including the signature catalytic triad (Cys-His-Asp/Asn) at the interface of its thumb and palm domains. A detailed structural comparison of the PLpro with UPS7 and USP2 proteins was carried out using the PDBeFold (SSM: Secondary Structure Matching) and multiple sequence alignment was performed with ESPript3 (Supplementary Figure 1). Although these proteins share a very low percentage of sequence identity with an alignment of only 187 residues of PLpro with USP7 and USP2 proteins, the structural superimposition topologies are reasonably similar with an overall root mean square deviation (RMSD) value of 1.68Å and 1.24 Å for USP7 and USP2, respectively (Figure 1A-C). Briefly, the palm subdomain of PLpro, USP7, and USP2 comprises of 6 β-sheets and includes the conserved catalytic triad (Cys-His-Asp/Asn) of the

active site (Figure 1A-C). Beyond the catalytic residues, USPs contain N- and C-terminal extensions that aid in substrate recognition and binding. The BL2 loop of SARS-CoV-2 PLpro (residues 267–272) that provides floor to ubiquitin tail, also superimposes well with the BL2 loops of USP7 and USP2 (Figure 1C) (Pozhidaeva et al., 2017; Wertz and Murray, 2019). Interestingly, the zinc-binding domain located in the thumb region of SARS-CoV-2 comprises of 4 cysteine residues (Cys189, Cys192, Cys224, and Cys226) and the same pattern is observed for USP2 as well (Cys334, Cys337, Cys381, and Cys384) (Renatus et al., 2006). Contrastingly, some of the cysteine residues of this metal binding motif have been mutated during evolution in USP7 and other DUBs due to which the zinc binding ability was lost while the fold integrity was still retained (Renatus et al., 2006). Importantly, residues Gly269, Gly271, His272, and Tyr273 (numbering as per PLpro) are conserved between USP7 and USP2 signifying that substrate binding or proteolytic cleft formed near the BL2 loop are also conserved (Figure 1 and Supplementary Figure 1) (Gao et al., 2021; Kiira et al., 2008). Nevertheless, the structural architecture of the active/catalytic site is very similar in PLpro, USP7, and USP2, suggesting that at least some inhibitors may display cross activity among these proteases.

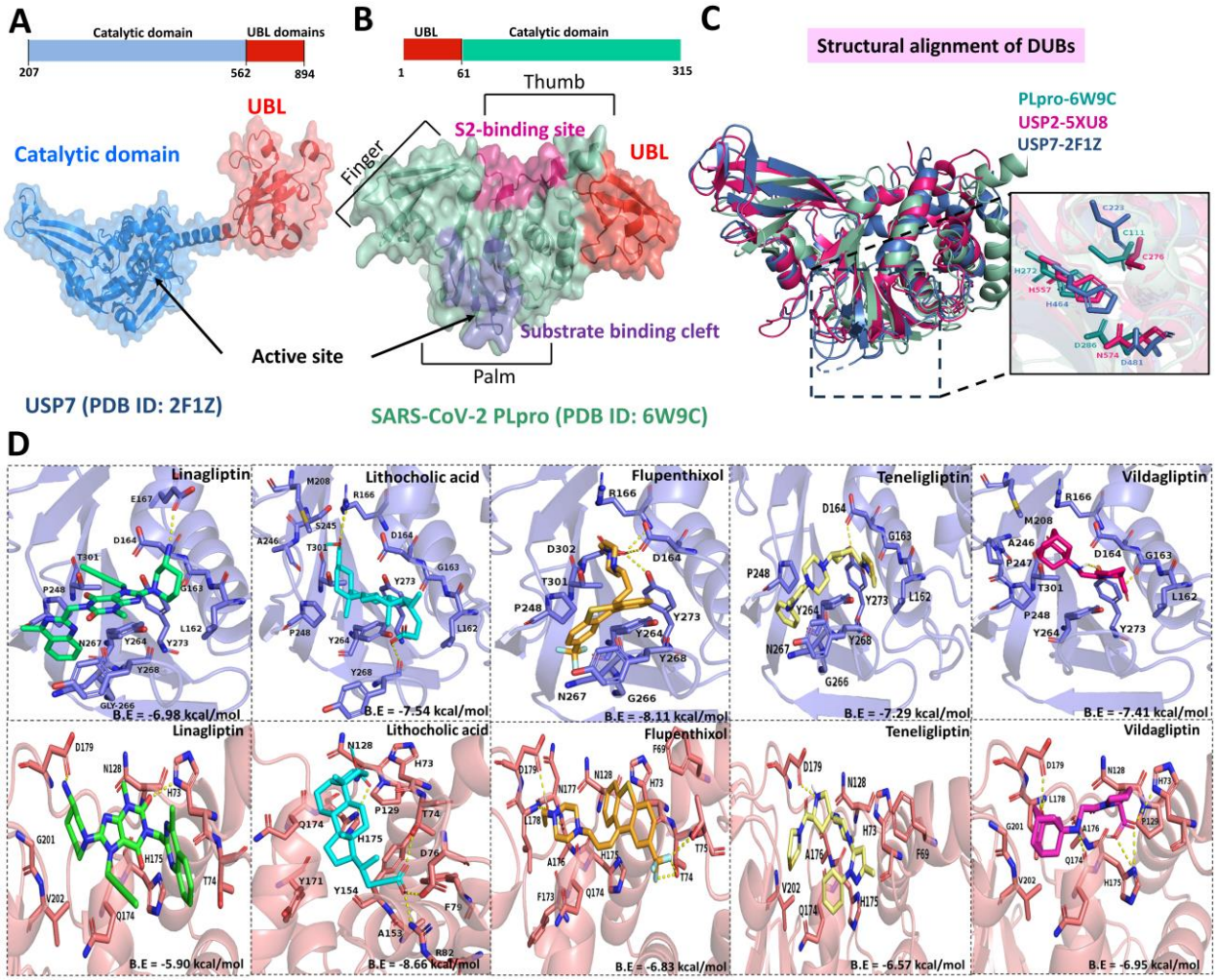

Figure 1. *Domain organization and comparison of structural motifs of SARS-CoV-2 PLpro with cellular DUBs.* ***(A)*** *Surface representation of the domain organization and structural architecture of USP7 (PDB ID: 2F1Z). The catalytic domain is represented in blue and the UBL domain is depicted as red surface.* ***(B)*** *Surface view representation of the overall structure of PLpro (PDB ID: 6W9C) with thumb, palm, and zinc-finger domains marked. The substrate binding cleft is depicted as violet surface, the S2 Ub/ISG15 binding site is represented as pink surface, and the UBL domain is shown as red surface.* ***(C)*** *Structural alignment of secondary structure elements of PLpro (Purple; PDB ID: 6W9C), with the cellular DUBs USP7 (Green; PDB ID: 2F1Z), and USP2 (Magenta; PDB ID: 5XU8). A total of 187 residues of each protein chosen by web-based server PDBeFold, were aligned based on the secondary structure elements. Cartoon representation shows PLpro in green, USP2 in pink, and USP7 in blue. The positioning of catalytic triad residues is represented in magnified view. Catalytic triad residues Cys, His, and Asp of PLpro, USP2, and USP7 represented in sticks, are completely superimposed.* ***(D)*** *PyMOL schematic representations demonstrating binding modes and key hydrophobic/hydrogen interactions of the top-hit screened ligands docked at the substrate-binding cleft (purple) and Ub/ISG15 S2-binding site (brick red) of PLpro.*

Structure-guided identification and binding mode analysis of PLpro-targeting compounds

Crystal structure of PLpro in complex with GRL0617, HE9, and acriflavine inhibitors provides a basis for identifying potential inhibitors targeting the multiple sites on SARS-CoV-2 PLpro (Gao et al., 2021; Napolitano et al., 2022; Srinivasan et al., 2022). To achieve this, an *in-house in silico* library of previously reported DUB inhibitors and cyanopyrrolidine ring-containing compounds, was subjected to high-throughput virtual screening protocol using an in-built AutoDock Vina module of PyRx 0.8 (Supplementary Table 1). A total of 20 compounds obtained after virtual screening were shortlisted based on binding affinities and RMSD values (<1 Å). The resulting scores of virtual screening for these 20 compounds are listed in Supplementary Table 2. Among these top hits, the compound Pimozide, MF094, FT671, ML323, FT827, GNE6640, and N-cyanopyrrolidine screened and identified in our study, were also reported recently to inhibit proteolytic activity of PLpro of SARS-CoV-2, thereby validating this developed protocol (Cho et al., 2022; Shan et al., 2021). Published literature highlights efficacy of sitagliptin and gemigliptin in type II diabetes mellitus patients infected with SARS-CoV-2 when administered as a monotherapy or in form of a combination therapy (Alhakamy et al., 2021; Hazra, 2021; Narayanan et al., 2022). These compounds were therefore omitted from further studies and the remaining compounds were processed to gain further insights into binding modes and stabilities of protein-ligand complexes. Based on binding energies, number of polar/hydrophobic interactions, and commercial availability, a total of five compounds were selected for detailed re-docking and simulation analysis using AutoDock4.2.6 and Gromacs.

The binding mode of GRL0617 (control molecule) with PLpro revealed an important hot-spot active site covering the residues Leu162, Gly163, Asp164, Glu167, Met208, Pro247, Pro248, Tyr264, Tyr268, Gln269, Gly271, and Tyr273, Thr301 that were targeted in this study for antiviral drug discovery (Fu et al., 2021; Klemm et al., 2020; Yuan et al., 2022). Importantly, PLpro efficiently deconjugates ISG15 (more preferentially) and ubiquitin modifications, dampening inflammation and antiviral signaling. The crystal structures of SARS-CoV-2 PLpro in complex with ISG15 (PDB: 6YVA) clearly demonstrates the binding of N-terminal region of ISG15 to a distal S2-binding site located on PLpro thumb domain, contributing to substrate recognition and differential cleavage (Figure 1, 6, and 7) (Gao et al., 2021; Shin et al., 2020). The C-terminal domain of ISG15 sits on the palm subdomain of SARS-CoV-2 PLpro allowing the C-terminal tail to reach the catalytic site. Key interacting amino acids mediating contacts of N-terminal ISG15 with S2 binding site of PLpro spans between 61-74, including Val66, Phe69, Glu70, and Thr75 (Patchett et al., 2021). Other contacts of S2-Ub or N-terminal ISG15 includes SARS-CoV-2 PLpro residues Asn128, Asn177, and Asp180 from the palm domain (Riva et al., 2020). The core S1-binding residues of PLpro mediating interactions with S1Ub or C-terminal ISG15 includes Asn156, Ser170, Tyr171, Gln174, Thr225, and Lys232 (Patchett et al., 2021). In conclusion, targeting the substrate-binding cleft and the S2 Ub/ISG15 binding sites of PLpro represents a new approach for preventing viral replication and its interaction with host cellular pathways.

Docking analysis revealed that the binding affinities of the top-screened compounds at the catalytic substrate-binding site ranged between -6.9 kcal/mol and -8.1 kcal/mol (Table 1). While the docking score for the control ligand GRL0617 is −6.94 kcal/mol. Docking studies of Flupenthixol revealed that the compound displays a high binding affinity of -8.11 kcal/mol and forms four hydrogen-bonds with active site residues Asp164, Arg166, Tyr273, and Asp302 (Figure 1D and Supplementary Figure 2). In addition, hydrophobic interactions between Flupenthixol and PLpro complex (Pro248, Tyr264, Gly266, Asn267, Tyr268, Thr301) also contributed to stabilization of this protein-ligand complex (Figure 1D). Lithocholic acid is the second-best candidate that interacts with three H-bonds with targeted amino acids Arg166 and Tyr268 along with ten hydrophobic interactions (Figure 1D and Table 1). Like other top hits, Teneligliptin and Linagliptin also occupied catalytic groove of PLpro forming H-bonds with key GRL0617 binding residues (Asp164: Teneligliptin; Glu167: Linagliptin) (Figure 1D and Supplementary Figure 2). The complexes of Teneligliptin and Linagliptin are further stabilized by extensive hydrophobic interactions primarily involving Leu162, Gly163, Pro248, Tyr264, Gly266, Asn267, and Tyr268 substrate binding residues of PLpro (Figure 1D and Supplementary Figure 2). Similarly, Vildagliptin also displayed a network of two H-bonds and ten hydrophobic interactions with the targeted site (Figure 1D, Supplementary Figure 2, and Table 1).

Due to the important role of S2-Ub/ISG15 binding site of PLpro, redocking studies were also conducted at this site and the data revealed binding affinities in the range of -5.9 to -8.6 kcal/mol. The compound Lithocholic acid, displayed highest binding energy when docked at S2 site,

interacted primarily with His73, Thr74, and Phe79, the key substrate interacting residues of S2 site (Figure 1D). Likewise, the compound Linagliptin interacted with His73 and Asp179 through H-bond interactions along with additional stabilizing hydrophobic interactions with key S2 site residues (Figure 1D). The compound Flupenthixol sustained seven H-bond interactions and other additional hydrophobic interactions with key interacting residues of S2 binding site such as, Phe69, His73, Asn128, Asn177 (Figure 1D). PLpro-Teneligliptin involved one H-bond with Asp179 and interacted with S2 site through additional hydrophobic interactions. The compound Vildagliptin was stabilized by six H-bond and additional hydrophobic interactions as shown in Table 1 (Figure 1D, Supplementary Figure 2).

To further assess the stability of docked complexes, Molecular dynamics (MD) simulation of the selected five compounds with PLpro was carried out for 100 ns (Supplementary Figure 3). The overall ligand/protein complex showed no major differences in the overall stability as observed for only PLpro, with an average RMSD value in the range of 0.1-0.4 Å for all complexes (Supplementary Figure 3). All complexes attained equilibrium after 20 ns and were stable throughout the run. Collectively, the data indicates that the selected compounds are valid starting points for optimization and development of new inhibitors against SARS-CoV-2.

Table 1. *Promising antivirals for PLpro of SARS-CoV-2 obtained after detailed molecular docking and MD simulation studies. Binding energies, hydrogen, and hydrophobic interactions are tabulated.*

Name of compound	Site	AutoDock Binding energies	Hydrogen bonds		Hydrophobic interactions
			Bonds (lig -protein)	Bond length (Å)	
Flupenthixol	Site1	-8.11	O-OD1 (Asp164)	3.17	Pro248, Tyr264, Gly266, Asn267, Tyr268, and Thr301
			O-N (Arg166)	3.16	
			O-OH (Tyr273)	2.95	
			O-OD2 (Asp302)	2.71	
	Site2	-6.83	F1-OG1 and F2-OG1 (Thr74)	2.69 and 2.6	Phe69, His73, Asn128, Phe173, Gln174, His175, Asn177
			F2-N and F2-OG1 (Thr75)	1.10 and 3.12	
			O-O (Ala176)	2.67	
			O-N (Leu178)	2.98	
			O-OD1(Asp179)	2.75	
Lithocholic acid	Site1	-7.54	O4-NE & O3-NH2 (Arg166)	2.87 and 3.05	Leu162, Gly163, Asp164, Met208, Ser245, Ala246, Pro248, Tyr264, Tyr273, and Thr301
			O2-O (Tyr268)	2.80	
	Site 2	-8.66	O1-OG1(Thr74)	2.81	His73, Phe79, Asn128, Pro129, Ala153, Tyr154, Tyr171, Gln174
			O2-OD2(Asp76)	2.86	
			O2-NH2(Arg82)	2.73	
			O3-ND1 (His175)	2.72	
Vildagliptin	Site1	-7.41	O4-O (Gly163)	2.55	Leu162, Asp164,

			N1-OH and O2-OH (Tyr273)	3.19 and 3.09	Arg166, Met208, Ala246, Pro247, Pro248, Tyr264, and Thr301
	Site 2	**-6.95**	O3-ND1 (His73)	3.14	Asn128, Pro129, Ala176, Gly201, Val202
			N1-O (Gln174)	2.74	
			O3-ND1 and O4-ND1 (His175)	2.98 and 3.16	
			O1-N (Leu178)	2.84	
			C4-OD1(Asp179)	2.71	
Teneligliptin	**Site1**	**-7.29**	N3-OD1 (Asp 164)	2.41	Leu162, Gly163, Pro248, Tyr264, Gly266, Asn267, and Tyr268
			N3-OH (Tyr 273)	2.78	
	Site 2	**-6.57**	N3-OD1(Asp179)	2.58	Phe69, His73, Asn128, Gln174, His175, Ala176, Val202
Linagliptin	**Site1**	**-6.98**	N27-OE1 (Glu167)	3.11	Leu162, Gly163, Asp164, Pro248, Tyr264, Gly266, Asn267, Tyr268, Tyr273, and Thr301
	Site 2	**-5.90**	O19-ND1 (His73)	3.03	Thr74, Asn128, Gln174, His175, Gly201, Val202
			N27-OD1 (Asp179)	2.59	

Compounds inhibits proteolytic activity of PLpro

Having established the enzymatic assay for proteolytic activity of PLpro, the inhibitory potential of five compounds selected from virtual screening and docking studies was quantified. To achieve this, recombinant SARS-CoV-2 PLpro was expressed in *Rosetta* DE3 and subsequently purified using affinity chromatography. A single band at ~36 kDa in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) signified pure PLpro protein (Figure 2A). Proteolytic activity of recombinantly purified PLpro was assessed by the optimized fluorescence resonance energy transfer (FRET) based protease assay, using the commercially available Z-RLRGG-AMC fluorogenic substrate representing the C-terminal residues of ubiquitin, as described in methodology section (Figure 2B). Purified PLproC111S mutant was used as a negative control to conclude that the production of fluorescence signal was exclusively dependent on proteolytic activity of enzyme (Figure 2A and 2B). The same FRET assay was used to assess and validate the inhibitory potential of the identified compounds, Flupenthixol, Lithocholic acid, Vildagliptin, Teneligliptin, and Linagliptin against the enzymatic activity of PLpro. Among these selected compounds, both Lithocholic acid and Linagliptin robustly inhibited proteolytic activity of PLpro with over 80% inhibition of SARS-CoV-2 PLpro and half-maximal inhibitory concentration (IC_{50}) values of 27.5 ± 2.20 μM and 53.1 ± 0.01 μM, respectively (Figure 2D and 2E). The compounds Flupenthixol and Teneligliptin showed IC_{50} values of 53.5 ± 4.09 μM and 259.2 ± 0.72 μM (Figure 2F and 2G). In contrast to it,

Vildagliptin was not observed to show any significant inhibition with a maximum inhibitory effect of less than 50 percent only (IC_{50} > 500 μM) (Figure 2C). Owing to its low inhibitory potential, Vildagliptin was not selected for Surface Plasmon Resonance (SPR) based binding kinetic assays and cell-based studies against SARS-CoV-2. Considering the inhibitory potential of the selected four compounds, these primary hits were subjected to a series of SPR-based assays and cell-based studies.

Surface Plasmon Resonance assay demonstrates binding of lead DUB inhibitors to PLpro

Prioritized lead compounds were tested in SPR assay to examine the binding kinetics and affinity of compounds against purified PLpro. After immobilization of histidine-tagged PLpro on Ni-nitrilotriacetic acid (Ni-NTA) chip, serial dose of selected compounds was allowed to flow over the chip and the time dependent optical signal was recorded. Consistent with the results of FRET assay, a dose-dependent increase in response was observed for binding of Lithocholic acid, Linagliptin, and Flupenthixol to SARS-CoV-2 PLpro. As represented in Figure 2H, the K_D values determined by SPR measurements for Lithocholic acid-PLpro, Linagliptin-PLpro, and Flupenthixol-PLpro are 79.3 μM, 72.6 μM, and 258.1 μM, respectively. SPR data suggested that Lithocholic acid and Linagliptin bind to PLpro with stronger affinity in comparison Flupenthixol (Figure 2H). While fitting the data for Teneligliptin, the derivatives of the binding response and the dissociation were nonlinear and attempts to fit the data with 1:1 Langmuir Binding model failed with Teneligliptin (data not shown). Together with results of fluorescence assay, SPR data confirms that these top-hits can be taken up for cell-based *in vitro* studies.

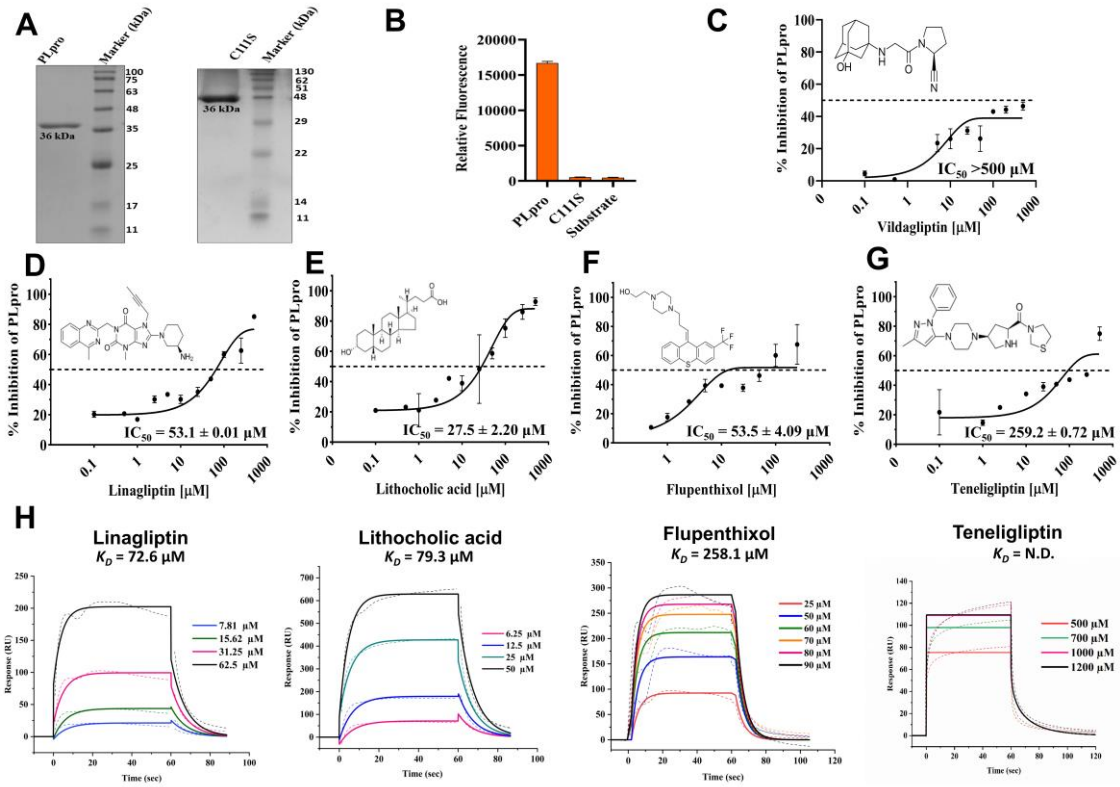

Figure 2. *Binding affinity analysis and inhibition of PLpro protease activity by selected compounds.* ***(A)*** *SDS-PAGE profile of purified PLpro (~36 kDa)* ***(B)*** *FRET based protease activity comparison of wild type and C111S mutant (inactive) PLpro. Inhibition of proteolytic activity of PLpro by selected compounds. The inhibitory potential of identified compounds* ***(C)*** *Vildagliptin,* ***(D)*** *Linagliptin,* ***(E)*** *Lithocholic acid,* ***(F)*** *Flupenthixol, and* ***(G)*** *Teneligliptin against PLpro were tested using fluorogenic peptide Z-RLRGG-AMC as the substrate. Black dotted lines indicate the concentration of compound needed to inhibit the proteolytic activity of PLpro by half (IC_{50}). All data are shown as mean ± SEM, n = 2 independent experiments* ***(H)*** *Evaluation of binding affinities of Lithocholic acid, Linagliptin, Flupenthixol, and Teneligliptin to SARS-CoV-2 PLpro using SPR. Sensorgrams elucidating the binding kinetics of selected analytes injected successively at increased concentrations against immobilized SARS-CoV-2 PLpro. The data was fitted using 1:1 Langmuir binding model. The processed sensograms are color coded, and the binding response is increasing with the increase in the concentration of the analyte. K_D, dissociation constant; RU, response units.*

Inhibitors abrogates replication of SARS-CoV-2 *in vitro*

To ascertain whether the selected compounds were cytotoxic or not, uninfected Vero cells, HEK-293T-ACE2, and A549-ACE2 were incubated with increased concentration of compounds and the cytotoxicity was measured using colorimetric MTT assay. As represented in Supplementary Figure 4, Lithocholic acid, Linagliptin, and Teneligliptin exerted very less cytotoxicity in all cell lines when tested across the range of concentrations as indicated (Supplementary Figure 4). Contrastingly, Flupenthixol displayed slight cytotoxicity at concentrations above 50 μM in the tested cell lines (Supplementary Figure 4). The data suggests safe cytotoxic profile of the selected compounds and qualified them for evaluation of antiviral potency against SARS-CoV-2.

Based on promising inhibitory activity of selected compounds against the PLpro, combined with their low cellular toxicity, we next sought to investigate their *in vitro* efficacy against clinical isolate of SARS-CoV-2 in Vero cells. Cells were pretreated with increased concentrations of compounds (Lithocholic acid, Linagliptin, Teneligliptin, and Flupenthixol) and were infected with SARS-CoV-2 at 0.01 MOI, followed by further incubation in medium with compound. Quantification of inhibition in viral replication was done by determining the percentage of positive infected cells in the presence and absence of the compound, at the end of the incubation period. To quantify this, cell lysate of treated and non-treated infected Vero cells was harvested to determine the viral load by quantifying the levels of viral RNA by real-time quantitative PCR (qRT-PCR), targeting the RdRp and N-protein. Consistent with the results of biophysical studies, Lithocholic acid and Linagliptin effectively inhibited replication of SARS-CoV-2 in Vero cells, exhibiting an EC_{50} value of 21 μM and 14.65 μM, respectively (Figure 3). Surprisingly, Flupenthixol, which was observed to be a relatively weak inhibitor of enzymatic activity of PLpro, also displayed a strong inhibitory effect against SARS-CoV-2 at its non-toxic concentrations with an EC_{50} value of 5.10 μM (Figure 3). Teneligliptin which was relatively a

promising inhibitor based on PLpro enzymatic assay also inhibited SARS-CoV-2 infection in Vero cells at low micromolar range (EC_{50} = 20.82 µM) (Figure 3).

To validate the results obtained from qRT-PCR, $TCID_{50}$ based studies were carried out. The effect of compounds on cells infected with SARS-CoV-2 was assessed by performing the virus titration. The sharp decrease in viral titer as compared to non-treated and virus infected positive control was observed in case of Linagliptin (decrease of 4 log $TCID_{50}$/mL) and Teneligliptin (decrease of 2.5 log $TCID_{50}$/mL) (Supplementary Figure 5). Flupenthixol at the concentration of 20 µM, decreased the viral titer by 1.5 log $TCID_{50}$/mL (Supplementary Figure 5) and Lithocholic acid (Supplementary Figure 5) at its highest concentration of 50 µM reduced the titer by 1 log $TCID_{50}$/mL as compared to virus control. We observed that treatment of cells with compounds involved in the present study, significantly reduced infectious virus production and cell death associated with virus infection. A concentration-dependent decrease in virus titer was observed in the case of all compounds (Supplementary Figure 5). Together, the data suggest that these molecules displayed dose-dependent inhibition of replication of virus in Vero cells and hold considerable promise for therapeutics against SARS-CoV-2.

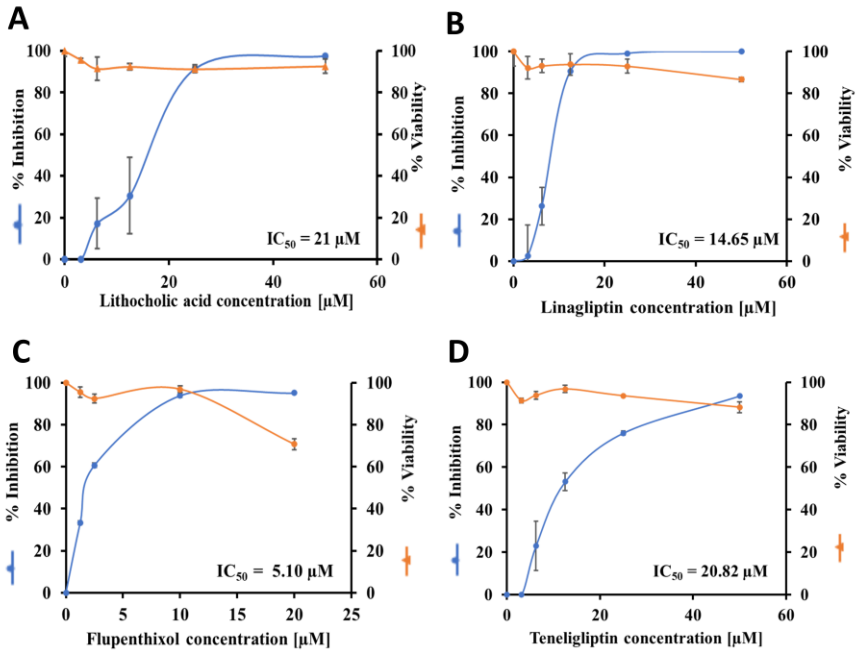

Figure 3. *Antiviral efficacy of selected compounds against SARS-CoV-2 infection in Vero cells.* ***(A-D)*** *Vero cells were treated with a series concentration of indicated compounds and were then infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01. Cell culture supernatant was harvested and the inhibition pattern was quantified by qRT-PCR at 48 hpi. Virus inhibition titer (EC_{50}) was estimated from dose–response curves of percentage inhibition versus compound concentration. Representative data are shown from duplicate readings and the final graph was*

plotted for percentage inhibition of cells in the presence and absence of compounds (n = 2). Data represent mean ± SD.

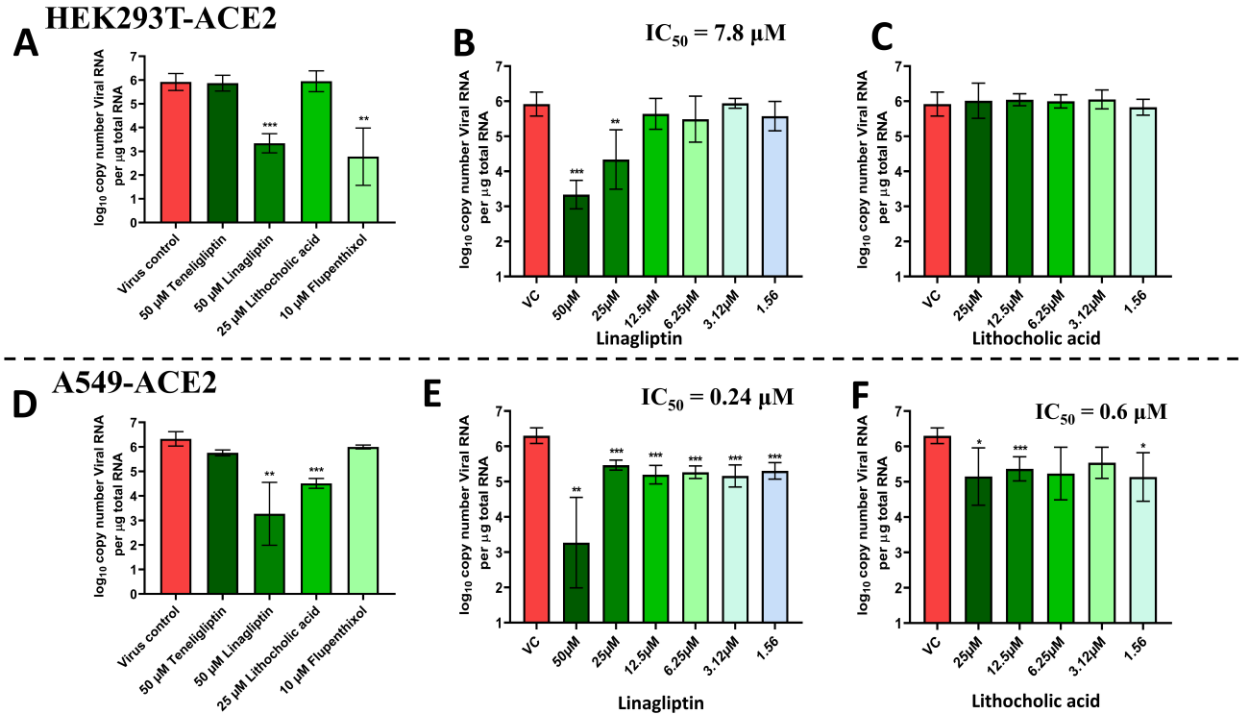

Figure 4. *Comparative antiviral efficacy of selected compounds against SARS-CoV-2 infection across multiple cell lines.* ***(A-F)*** *Cells were pre-treated with indicated drug concentrations for 3 h, washed and infected with 0.01 MOI SARS-CoV-2 for 2 h in the absence of drugs. After further washing, cells were infected with infection media containing drugs and 48 h later, viral RNA load was estimated from total cellular RNA by qRT PCR.* ***(A)*** *shows results for HEK293T-ACE2 cells treated with single non-toxic concentrations of Teneligliptin, Linagliptin, Lithocholic Acid or Flupenthixol,* ***(B, C)*** *show data for increasing concentrations of Linagliptin and Lithocholic acid respectively.* ***(D-F)*** *represent similar datasets in A549-ACE2 cells. Results represent data from 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 using Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparison tests. Error bars represent mean ± SD.*

Validation of inhibitory effects in human cell lines

To ensure that the observed antiviral efficacies of selected compounds were not restricted to Vero cells, the compound efficacies were further evaluated on two additional human cell lines expressing ACE2, HEK293T-ACE2 and A549-ACE2 that supports SARS-CoV-2 replication. Cytotoxicity assay was performed in both HEK293T-ACE2 and A549-ACE2 cells to determine the working concentrations of the compounds. Lithocholic acid was toxic in HEK293T-ACE2

cells at a concentration of 25 µM, and Flupenthixol at 20 µM concentration led to ~20% loss in cell viability in both cell lines (Supplementary Figure 4). Based on this, the following highest non-toxic doses were chosen for the drugs: 50 µM Teneligliptin, 50 µM Linagliptin, 25 µM Lithocholic acid, and 10 µM Flupenthixol.

Antiviral assays were performed using highest non-toxic doses of the four drugs. Dose-titration analysis revealed that nearly all (3 out of 4) tested compounds inhibited replication of SARS-CoV-2 in one or both of these cell lines at potencies equivalent to or greater than those that was observed in case of Vero cells. Results showed consistent inhibition of vRNA loads by a scale of ~ 3 $\log_{10}$, in the presence of 50 µM Linagliptin in HEK293T-ACE2 (IC_{50}= 7.4 µM) and A549-ACE2 (IC_{50}= 0.24 µM) cells respectively (Figure 4A, B, D, E). Lithocholic acid did not show any antiviral effects in HEK293T-ACE2 cells, but inhibited viral load by ~1.5 $\log_{10}$ (IC_{50} = 0.6 µM) in A549 ACE2 cells (Figure 4A, C, D, F). Flupenthixol inhibited viral load by ~ 3 $\log_{10}$ in HEK293T-ACE2 cell line but failed to show any effect in A549-ACE2 cells (Figure 4A, D). A possible reason for the differential efficacy of some compounds in different cell lines could be species-specific or cell-type-specific mechanism of action of a given compound or by differences in drug metabolism (Biering et al., 2021; Riva et al., 2020).

Effects of compound treatment on cytokine expression and IFN responses

Infection by SARS-CoV-2 induces a hyper-inflammatory response driven by the uncontrolled release of TNF-α, IL-6, IL-1, IL-8, CXCL10 or delayed type I interferon response. Therefore, to understand the effects of identified compounds on the expression levels of proinflammatory cytokines induced by SARS-CoV-2, the mRNA expression levels of cytokines were quantified in presence of compounds using qRT-PCR. The data suggests that the compounds Lithocholic acid and Linagliptin were observed to inhibit the expression of IL6 (to a greater extent) and TNFα in HEK293T cells stimulated by polyinosinic:polycytidylic acid (poly I:C), a synthetic analog of dsRNA that mimics virus infection (Figure 5A and B). This indicates that treatment with Lithocholic acid or Linagliptin could have an effect on virus induced inflammatory responses (Figure 5A and B). The PLpro of SARS-CoV-2 antagonizes the type-1 IFN innate immune responses of the host cell reducing the amounts of phosphorylated and ISGylated IRF3 or after deubiquitination of stimulator of interferon genes (STING) (Cao et al., 2023; Chen et al., 2014; Yuan et al., 2022). Consistent with published findings, the results of this assay also revealed that PLpro of SARS-CoV-2 suppressed the levels of IFNβ (Figure 5C)(Crudele et al., 2022; Shin et al., 2020). Further analysis revealed that the compounds Lithocholic acid and Flupenthixol were able to restore the levels of interferon-beta (IFNβ) in HEK293T cells transfected with SARS-CoV-2-PLpro (Figure 5C). By contrast, no effect was observed on the expression levels of IFNβ when cells were treated with Linagliptin and Teneligliptin (Figure 5C).

To confirm whether the restored levels of IFNβ is due to inhibition of PLpro, a luciferase reporter assay was used to test the effects of drugs on type-1 interferon induction using an IFNβ promoter-driven firefly luciferase reporter plasmid (IFN-Beta_pGL3) and a control plasmid which constitutively expresses renilla luciferase gene (pRL-TK). RIG-I 2CARD was used as an inducer of type-1 IFN induction at a very early step of the signaling pathway upstream of nuclear translocation of IRF3 and expression of interferon stimulated genes (ISGs). The plasmids were co-transfected with plasmid expressing SARS-CoV-2 PLpro to test the effect of the drugs to rescue the viral protein induced inhibition of IFN-β production. Having demonstrated a role for expression of SARS-CoV-2 PLpro in attenuating host antiviral IFN pathways, it was anticipated that inhibition by identified compounds would reverse this process. Interestingly, treatment with Lithocholic acid caused ~4-fold increase of IFN-β production signifying that the compound was able to restore the suppressed type 1 interferon responses (Figure 5D). Indeed, none of the drugs could rescue the PLpro induced IFN inhibition (Figure 5E).

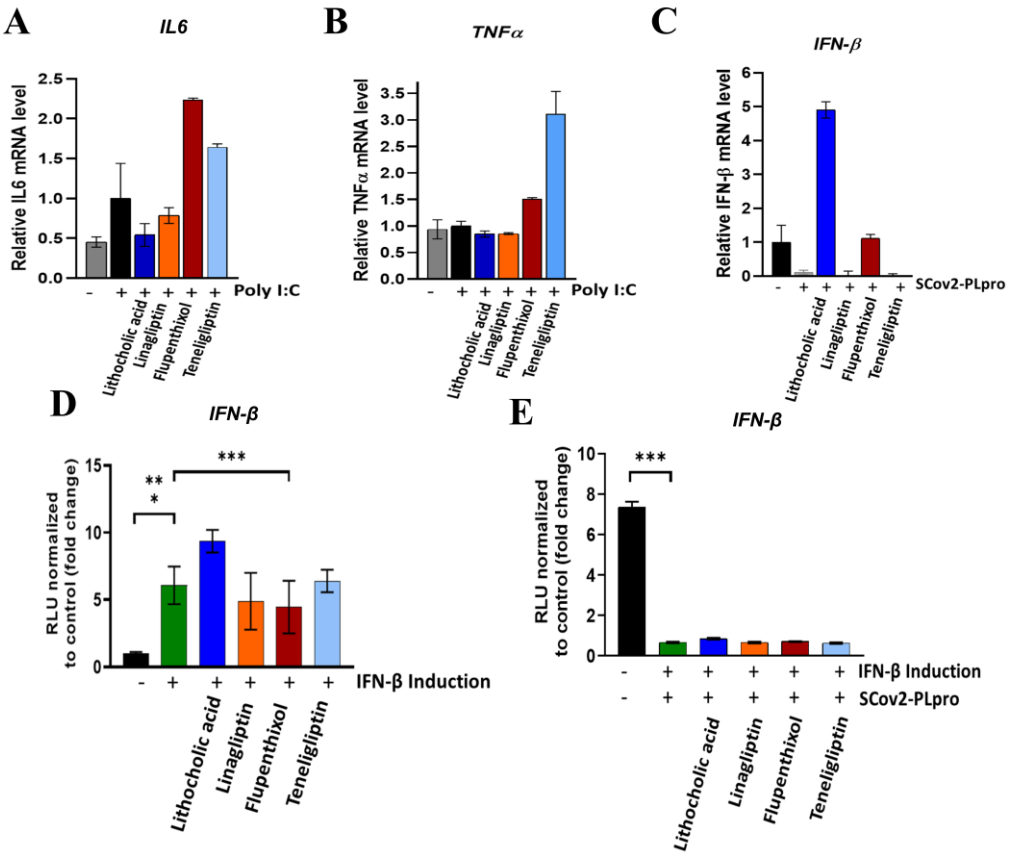

Figure 5. *Effects of compound treatment on inflammatory cytokines and delayed type I interferon response.* ***(A, B)*** *HEK293T cells were stimulated with Poly I:C for 6 h and were treated with 30 µM of Lithocholic acid (blue), 30 µM of Linagliptin (orange), 10 µM of Flupenthixol (red), and 30 µM of Teneligliptin (light blue).* ***(C)*** *For evaluation of IFN-β levels,*

the cells were transfected with plasmid encoding SARS-CoV-2 PLpro followed by stimulation with poly I:C and treatment with compounds. RNA was isolated after 24 h and gene expression analysis for cytokines was performed using qRT-PCR. ***(D, E)*** *HEK292T cells were transfected with IFNβ-luc firefly luciferase reporter plasmid and pRL-TK Renilla luciferase reporter plasmid, along with plasmid expressing SARS-CoV-2 PLpro or empty vector. RIGI 2CARD was used as an inducer of type-1 interferon production. 3 h post transfection, cells were treated with different drugs 25 µM of Lithocholic acid, 50 µM of Linagliptin, 10 µM of Flupenthixol, and 50 µM of Teneligliptin, and 24 h later, cells were collected, and luciferase expression was analyzed by dual luciferase assay.* ***(D)*** *shows results for the effects of drugs on IFN-β promoter activity.* ***(E)*** *shows effects of drugs in rescuing SARS-CoV-2 PLpro induced inhibition of IFN-β production. Results represent data from 2 independent experiments. ***p < 0.001; using One-way ANOVA with Dunnett's T3 multiple comparison tests. Error bars represent mean ± SD.*

X-ray crystal structures of SARS-CoV-2 PLpro in complex with inhibitors

We next set out to determine crystal structures of PLproC111S in complex with identified inhibitors (Lithocholic acid, Linagliptin, Teneligliptin, and Flupenthixol) to gain comprehensive insights into the molecular mechanisms by which these inhibit the proteolytic activity of PLpro and further SARS-CoV-2 in cell-based conditions. The data for complex structures of PLproC111S:Linagliptin and PLproC111S:Lithocholic acid were solved in $P3_221$ space group at a resolution of 2.7 Å and 2.3 Å, respectively with one biologically active monomer in the asymmetric unit (Table 2).

Analysis of the electron density maps signifies that Linagliptin accommodates the ISG15/Ub S2-binding site of PLpro, near the thumb domain, consequently creating sterical hindrance and blocking the N-terminal domain of these substrates from accessing the S2 site (Figure 6A-C).). In-depth structure analysis reveal that, Linagliptin interact with the side chains of surrounding residues, including Phe69, His73, Gln174, Ala176, Leu178, and Asp179 through hydrophobic interactions (Figure 6B). The side chains of Asn128, Gln174, and His175 is involved in H-bonding with the xanthine and purinedione skeleton of linagliptin which occupies the S2-site (Figure 6C). Interestingly, the 3-amino-piperidine ring and the purinedione skeleton of linagliptin bind to key residues Phe69, His73, and Asn128 within the S2 Ub/ISG15-binding pocket of PLpro, the core residues mediating hydrophobic interactions with ISG15 and ubiquitin substrates (Figure 6A, and B) (Békés et al., 2016; Klemm et al., 2020; Wydorski et al., 2023). Superimposition of PLproC111S:Linagliptin complex structure with PLpro-ISG15 complex (PDB ID: 6YVA previously reported) in turn prove that Linagliptin occupies the Ub/ISG15 binding pocket on PLpro, and disrupts the protein-protein interaction between PLpro and ISG15/Ub substrates (Figure 6B and C). After binding of Linagliptin, the critical residues Ser22, Met23, and Glu27 available on the binding surface of ISG15, are no longer able to access the S2 pocket of PLpro to make connections with PLpro (Figure 6B). Indeed, these lost interactions of

ISG15 to PLpro also supports the notion that the compound acts by disrupting the protein-protein interaction (PPI) between PLpro and Ub/ISG15.

A difference Fourier map contoured at 3σ allowed unambiguous tracing of Linagliptin at the S2 site, except the terminal ring of quinazoline moiety. Despite multiple attempts for X-ray diffraction and data collection, a complete electron density for this ring was unable to be obtained. This prompted an evaluation of the quality, purity, and the structural intactness of Linagliptin (purchased commercially) using High-Performance Liquid Chromatography (HPLC), along with assessment of the effects of crystallization buffers on integrity of this compound. HPLC analysis revealed that Linagliptin displayed a single elution peak at similar retention time ~3.2 min and the UV spectra gave peaks at ~230 nm proving the purity and intactness of the compound compared to standards in control (stock solution) for all reaction conditions (Linagliptin incubated in crystallization buffer at different time points), suggesting that the crystallization buffers have no impact on the structural intactness of the compound (Supplementary Figure 6). In conclusion, a slightly weak electron density of this aromatic ring could presumably be due to a high flexibility of this moiety (Davis et al., 2003).

Table 2. *Data collection and refinement statistics.*

	8XTD (PLpro-Linagliptin)	**8X1X (PLpro-Lithocholic acid)**
Resolution range	23.09 - 2.7 (2.79 - 2.7)	24.84 - 2.3 (2.38 - 2.3)
Space group	P 3_2 2 1	P 3_2 2 1
Unit cell a, b, c (Å) **α, β, γ (°)**	81.2 81.2 133.4 90 90 120	81.7 81.7 133.6 90 90 120
Total reflections	223494 (16276)	203675 (12653)
Unique reflections	14478 (1412)	23557 (2319)
Completeness (%)	99.7 (99.8)	99.75 (99.96)
Mean I/σ(I)	15.5 (1.8)	29.13 (2.59)
R-merge	0.2 (1.3)	0.454 (0.61)
CC1/2	0.9 (0.7)	0.884 (0.53)
Reflections used in refinement	14471 (1412)	23532 (2321)
Reflections used for R-free	684 (40)	1202 (116)

R-work	0.2	0.21
R-free	0.28	0.25
Number of non-hydrogen atoms	2588	2767
macromolecules	2502	2531
ligands	71	100
solvent	15	136
Protein residues	315	315
RMS (bonds)	0.01	0.01
RMS (angles)	2.2	2.42
Ramachandran favored (%)	90.7	86.82
Ramachandran allowed (%)	7.6	9.00
Ramachandran outliers (%)	2.0	4.0
Rotamer outliers (%)	8.73	9.25
Average B-factor	45.3	37.54
macromolecules	44.2	36.42
ligands	86.9	69.72
solvent	29.5	34.85

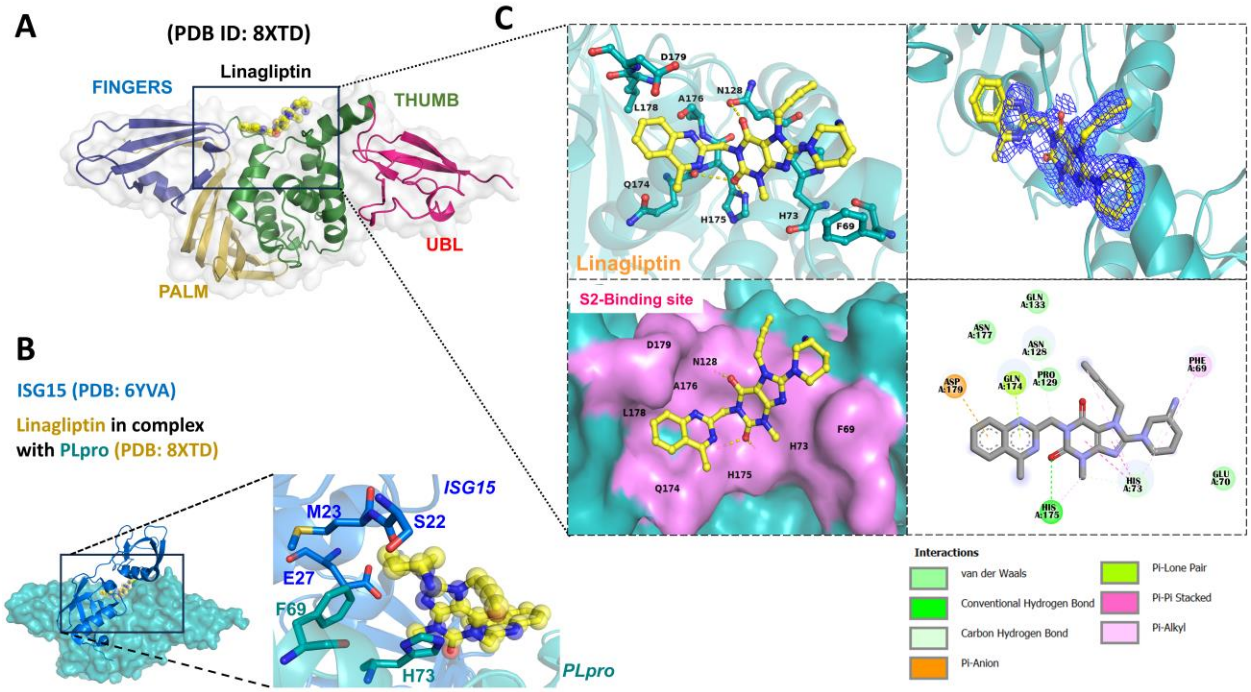

Figure 6. (A) *Crystal structure of SARS-CoV-2 PLpro in complex with identified compound Linagliptin (PDB ID: 8XTD) with PLpro shown as cartoon representation and the Linagliptin shown as yellow spheres. The identified compound Linagliptin binds at the Ub/ISG15 S2-binding pocket. The thumb domain (residues 61-180) is colored as green (residues 61-180), the palm (residues 239-315) as yellow, the finger (residues 181-238), and the UBL domain (residues 1-60) as red.* ***(B)*** *Superimposition of the crystal structure of SARS-CoV-2 PLpro:Linagliptin (PDB ID: 8XTD) complex with crystal structure of SARS-CoV-2 PLproC111S in complex with mouse-ISG15 (PDB ID: 6YVA, ISG15 in blue cartoon). SARS-CoV-2 PLpro is depicted as teal green and the identified compound Linagliptin is represented as yellow spheres.* ***(C)*** *An enlarged view depicting intermolecular interactions of Linagliptin to the S2-binding pocket of PLpro. The compound is shown as yellow sticks and the PLpro residues involved in binding are depicted as teal green sticks. Dashed yellow lines show hydrogen bonds. The electron density Fo-Fc map (contour level = 3 σ) is shown as blue mesh. The 2-dimensional image for PLpro-Linagliptin interactions is prepared using Discovery Studio.*

To understand the molecular basis of SARS-CoV-2 PLpro inhibition by Lithocholic acid, the X-ray crystal structure for protease co-crystallized with Lithocholic acid was determined (PDB ID: 8X1X). The structure revealed electron density of Lithocholic acid occupying three binding sites on PLpro, including the substrate binding-cleft near the BL2 groove and the Ub/ISG15 S2-binding pocket (Figure 7). At the S2-binding pocket, Lithocholic acid I form hydrophobic interactions with the side chains of Phe69, His73, Asn128, Gln174, His175, Ala176, Asn177, Leu178, and Asp179 (Figure 7B and C). Interaction of Lithocholic acid I with the key natural substrate interacting residues of PLpro (Phe69, His73, Asn128) reveals molecular basis of

inhibition mechanism of PLpro by Lithocholic acid I, disrupting the PPI between PLpro and ISG15 or di-Ub (Figure 7B and C). Superimposition of PLproC111S:Lithocholic acid I complex structure over SARS-CoV-2 PLpro:ISG15 structure suggests disruption of interactions between PLpro and ISG15. Remarkably, in the presence of Lithocholic acid I, the essential residues of ISG15, Ser22, Met23, Ser26, and Glu27, which are known to interact with the S2 pocket of PLpro, are no longer accessible to form contacts with PLpro (Figure 7B). To our surprise, a second binding site for Lithocholic acid II was observed at a novel site near the zinc-finger motif (Figure 7C). The interactions of inhibitor are stabilized by hydrophobic interactions with Tyr213, Lys217, Thr257, Tyr305, Lys306, Glu307, Asn308, and Ser309 (Figure 7C). Additionally, Lithocholic acid II forms H-bond interaction with Glu214 further stabilizing the complex (Figure 7C).

In addition, a third molecule of Lithocholic acid III, occupies the substrate-binding cleft pocket forming interactions with Asn267 and Gln269, the two prime residues of the flexible BL2 loop. Conformational changes in the BL2 loop are reported to regulate the binding, substrate accessibility, and enzymatic activity of PLpro. Asn267 and Gln269 are reported to accommodate the C-terminal leucine residue at P4 position of PLpro substrates. Interestingly, analysis of the BL2 loop suggests that upon binding with Lithocholic acid III, the main chain atom of Asn267 forms H-bond with inhibitor. The ligand is presumed to stabilize the inhibited state of PLpro after establishing relevant interactions with Gln269 (BL2-loop) and Asn267 (substrate binding site). A similar set of interactions were previously observed in other reported crystal structures of PLpro (PDB ID: 7LBR, 7JIR, and 7JIW) and specific interaction with Asn267 and Gln269 was shown to be essential for inhibition. The binding of Lithocholic acid does not induce significant conformational changes in the BL2 when compared with unliganded apo form of PLpro (PDB ID: 6WZU). The most dramatic change was observed at the residue Gln269. In the apo structure, the side chain of Gln269 swings away from the pocket. In the Lithocholic acid-bound structure, the side chain of Gln269 rotates inwards towards the substrate-recognition cleft, thus narrowing the substrate-binding cleft and clamping the inhibitor to the protease. The crystallographic data and refinement statistics are listed in Table 2.

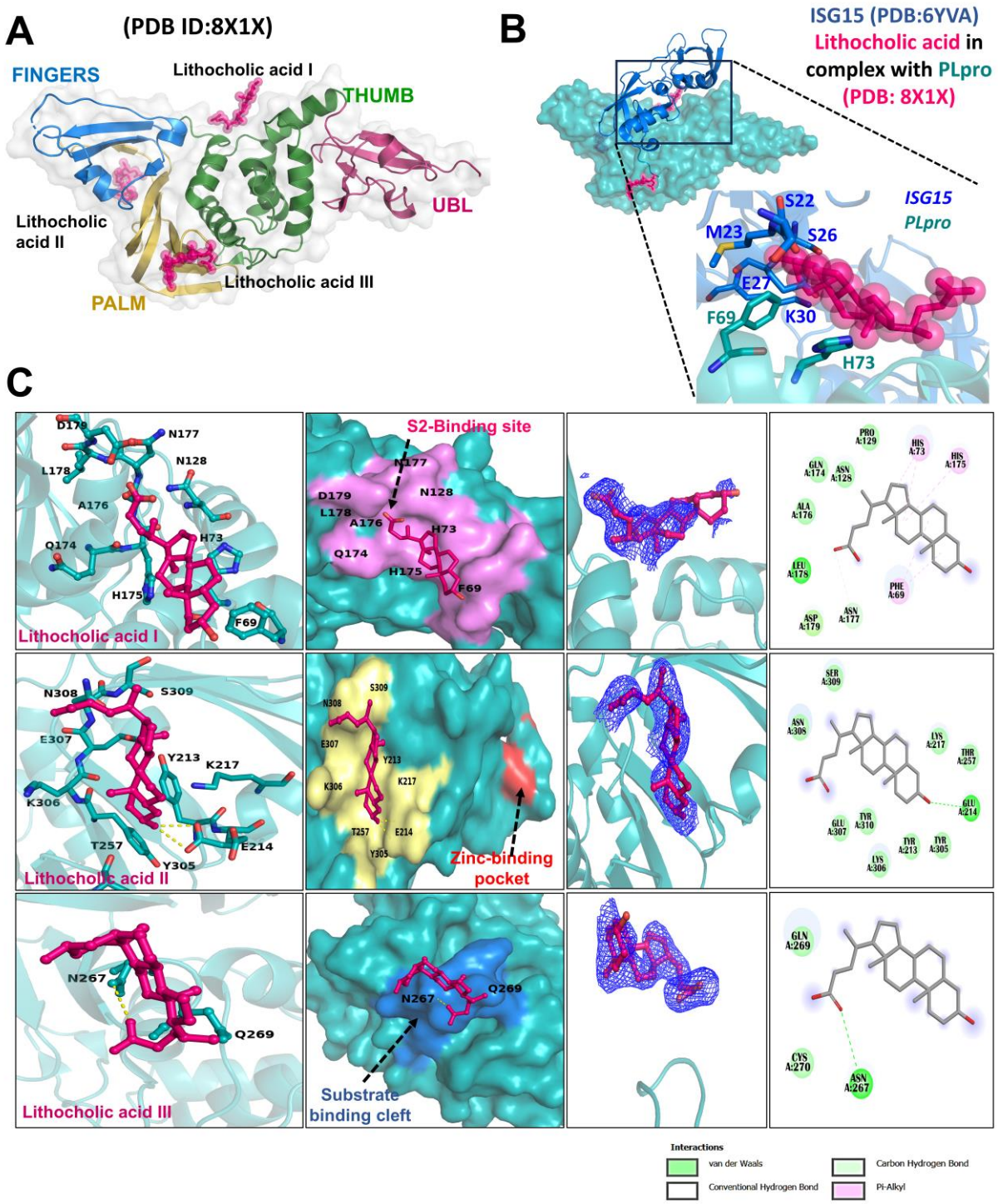

Figure 7. (A) *Crystal structure of SARS-CoV-2 PLpro-C111S in complex with identified compound Lithocholic acid with PLpro shown as cartoon representation and the Lithocholic acid shown as pink spheres. The identified compound binds at three sites: Lithocholic acid I (Ub/ISG15 S2-binding site), II (a novel site near the zinc-binding pocket), and III (Substrate binding cleft). The thumb domain (residues 61-180) is colored as green (residues 61-180), the*

palm (residues 239-315) as yellow, the finger (residues 181-238), and the UBL domain (residues 1-60) as red. ***(B)*** *Superimposition of the crystal structure of SARS-CoV-2 PLpro:Lithocholic acid complex with crystal structure of SARS-CoV-2 PLproC111S in complex with mouse-ISG15 (PDB ID: 6YVA, ISG15 in blue cartoon). SARS-CoV-2 PLpro is depicted as teal green surface and the identified compound Lithocholic acid is represented as pink spheres at three sites.* ***(C)*** *An enlarged view depicting intermolecular interactions of Lithocholic acid I to the S2-binding pocket, Lithocholic acid II to a novel site near the zinc-binding pocket, and Lithocholic acid III to substrate-binding pocket. The compound is shown as pink sticks and the PLpro residues involved in binding are depicted as teal green sticks. Dashed yellow lines show hydrogen bonds. The electron density Fo-Fc map (contour level = 3 σ) is shown as blue mesh. The 2-dimensional images for PLpro-Lithocholic acid interaction are prepared using Discovery Studio.*

Linagliptin exhibits *in vivo* antiviral efficacy in mouse model of SARS-CoV-2 infection

Having identified Linagliptin as the lead compound with the most promising results in biophysical assessments and *in vitro* anti-SARS-CoV-2 studies, the *in vivo* antiviral efficacy of this compound was investigated using the mouse-adapted SARS-CoV-2 model (MA10). To this end, mice were intranasally infected with 1×10^5 PFU of mouse- adapted SARS-CoV-2 (MA10) 6 h before the first dose. Challenged mice received treatments with Linagliptin twice daily for 4 days via intraperitoneal (i.p.) and oral (p.o.) routes at doses of 30 mg/kg and 60 mg/kg. Molnupiravir was used as a positive control and dosed orally and intraperitoneally at 100 mg/kg and 200 mg/kg. SARS-CoV-2 lung titers were quantified for the treatment group and were compared to vehicle and molnupiravir control (Figure 8B). Overall, both i.p and oral administration of Linagliptin (30 mg/kg and 60 mg/kg) displayed promising antiviral efficacy of Linagliptin against SARS-CoV-2 with more effective outcomes in oral administration than the intraperitoneal route (Figure 8B and C). The antiviral effect of Linagliptin was evident with over a log viral titer reduction in lung viral load (60 mg/kg i.p and 30 mg/kg oral) relative to the control infected groups (Figure 8B). Histopathological analysis revealed that oral administration of Linagliptin (30 mg/kg and 60 mg/kg) significantly reduced the severity of lung lesions, including peribronchiolar and perivascular infiltration, bronchiolar epithelial desquamation, and alveolar septal infiltration, compared to the control infected groups (Figure 8C and D).

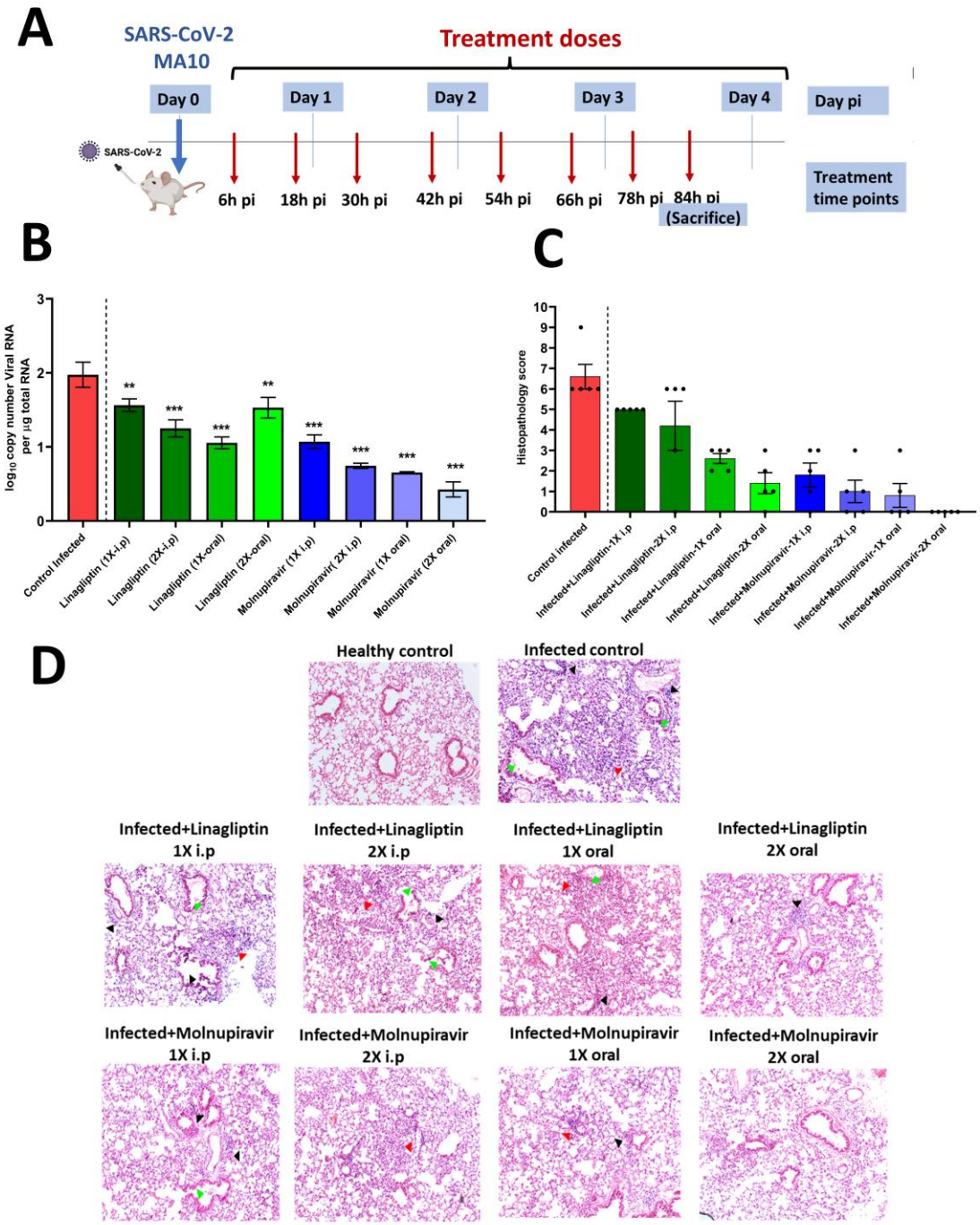

Figure 8. *Linagliptin shows in vivo antiviral efficacy in mouse-adapted model of SARS-CoV-2 (MA-10).* ***(A)*** *A schematic view of the experimental plan. Female mice were dosed with two different concentrations of Linagliptin and Molnupiravir starting from 6h post infection until the day of sacrifice.* ***(B-C)*** *On day 4 post infection, lungs were harvested for estimating viral RNA load and histology analysis.* ***(D)*** *Histopathological scoring of the lung tissue. Lung histopathology lesions (10X) showing peribronchiolar and perivascular infiltration (black arrow*

head), bronchiolar epithelial desquamation (green arrow head) and alveolar septal thickening (red arrow head).

Discussion

The rapid rollout of vaccines played a crucial role in battling the pandemic of COVID-19 (Li et al., 2022; Mello et al., 2022). However, the rise of emerging variants, breakthrough infections, and drug resistance underscores the critical need for orally bioavailable broad-spectrum antivirals as a part of pre-preparedness plan. Drug repurposing provides an alternative approach for quick identification of potential drugs to combat COVID-19, and targeting PLpro offers additional broad-spectrum advantages owing to its druggability and highly conserved substrate-binding pocket. Several host proteases, including USP2 and USP7 exhibit the same deconjugating activity and structural homology as host-encoded DUBs, marking their similarity as a noteworthy target to be explored (Figure 1). In addition to the protease activity, PLpro possesses DUB and deISGylating activities as an evasion mechanism against the host antiviral immune response. To evade host immunity, PLpro antagonizes ISG15 dependent activation of MDA5 and RIG1 protein through its DUB activity (Liu et al., 2021). A dysregulated interferon mediated antiviral response could lead to pro-inflammatory cytokine storm in the COVID-19 patients, a major cause of mortalities among infected patients (Liu et al., 2021). Thus, targeting PLpro might serve as a multifaceted approach that could avert SARS-CoV-2 infection either by inhibition of its proteolytic activity or by upregulation of innate immune response.

In search of a broad-spectrum antiviral drug, the present study investigated the antiviral efficacy of DUB inhibitors against PLpro, owing to the structural and functional similarities (Figure 1). Structure-based drug repurposing revealed five promising molecules targeting the Ub/ISG15 S2-binding pocket and the catalytic site of PLpro, from an *in-house* library of DUB inhibitors (Table 1, Supplementary table 1, and Figure 1). The enzymatic assay and SPR binding kinetic studies against PLpro demonstrate that Lithocholic acid, Linagliptin, Teneligliptin, and Flupenthixol hold tremendous promise as a new class of antivirals against PLpro (Figure 2). Interestingly, ligand-binding experiments using SPR were in line with the enzymatic inhibition studies with observed K_D values in the micromolar range (Lithocholic acid -PLpro: 79.3 μM; Linagliptin-PLpro: 72.6 μM; Flupenthixol-PLpro: 258.1 μM) (Figure 2). The selected compounds efficiently inhibited not only the proteolytic roles of PLpro but also the viral replication (Figure 2 and 3). Concentration-dependent reduction in viral copy number and infectious viral titer well below their cytotoxic concentration highlights the effective anti-SARS-CoV-2 activity of compounds in the initial experiments in Vero cells (Figure 3). Dose-titration data depicts that nearly all (3 out of 4) tested compounds significantly inhibited replication of SARS-CoV-2 in one or both HEK293T-ACE2 and A549-ACE2 cell lines at potencies equivalent to or greater than those that was observed in case of Vero cells (Figure 4). Though Teneligliptin failed to discern binding with PLpro in SPR assay, its antiviral data suggested significant inhibitory effect against SARS-CoV-2, proposing a

hint for other possible off-targets that could be explored (Figure 2 and 3). Linagliptin demonstrated the strongest inhibition in both A549-ACE2 and HEK293T-ACE2 cells with EC_{50} value of 0.24 and 7.4 μM, respectively (Figure 4). The cytotoxicity profile in Vero cells, HEK293T-ACE2, and A549-ACE2 showed a CC_{50} value of > 50 μM marking them safe for use (Supplementary Figure 4).

To obtain further mechanistic understanding of the mode of inhibition, structures of Linagliptin and Lithocholic acid in complex with PLproC111S were determined using X-ray crystallography (Figure 6 and 7). The data indicates that both Linagliptin and Lithocholic acid occupied the Ub/ISG15 S2-binding pocket of PLpro at the thumb domain, located at the interface between PLpro and ISG15 (Figure 6 and 7). Once Linagliptin or Lithocholic acid occupies the S2 interface between PLpro and ISG15 substrate, it is likely to disrupt the interaction between PLpro and natural substrates such as ISG15 or di-Ub. Furthermore, by blocking the access of Ub/ISG15 to the S2 binding pocket on PLpro, Linagliptin or Lithocholic acid offers potential to downregulate the deubiquitinase or deISGylase activity, thereby providing a novel strategy for structure-based inhibition of SARS-CoV-2 and its emerging variants (Figure 6 and 7). Of all the compounds tested, Linagliptin exhibited significant inhibitory effects in biophysical, biochemical, and virus inhibition across multiple cell lines, and was thus selected as prime candidates for *in vivo* investigation in intranasally infected mouse-adapted MA10 SARS-CoV-2 model (Figure 8). Twice-daily oral and intraperitoneal administration of Linagliptin (30 mg/kg and 60 mg/kg of body weight) to mice for 4-day period significantly reduced the lung viral RNA load, reduced weight loss, and ameliorated SARS-CoV-2 induced histopathological damages *in vivo* (Figure 8).

Previous studies on Lithocholic acid report it to be a potent inhibitor of intestinal inflammatory responses (Ward et al., 2017). In another study, Lithocholic acid is reported to exert antiviral action on replication of porcine delta coronavirus replication (PDCoV) (Kong et al., 2021). Recent clinical and experimental data on COVID-19 provides evidence for reduced mortality in infected patients with or without type 2 diabetes, after the use of gliptins (e.g., sitagliptin, alogliptin, etc.). Gliptins, a class of DPP4 inhibitors, antagonize SARS-CoV-2 virulence either by enhancing GLP-1 anti-inflammatory activity or by reducing overproduction of cytokines and downregulating the activity of macrophages (Strollo and Pozzilli, 2020)(Solerte et al., 2020). These classes of inhibitors may halt the disease progression to a hyperinflammatory state after viral infection.

In conclusion, the compounds identified in this study offer new perspectives for treatment of COVID-19. Given that the identified PLpro inhibitors acts through a novel mechanism, follow up characterization of these inhibitors in combination with Mpro and RdRp inhibitors may help to further mitigate the risk of drug-resistant viral evolution and enhance pandemic preparedness. Overall, Linagliptin is a strong therapeutic candidate for further development as an oral anti-SARS-CoV-2 antiviral.

Material and Methodology

Cells, viruses, and compounds

Vero and HEK293T cell line used in this study were procured from NCCS (Pune, India). The cells were maintained in high glucose Dulbecco's-modified essential media (DMEM; HiMedia, India) augmented with 10% heat inactivated fetal bovine serum (FBS; Gibco, USA), 100 units of penicillin, and 100 mg streptomycin/mL. HEK293T cells expressing human ACE2 (HEK293T-ACE2) (NR-52511, BEI Resources, NIAID, NIH) and A549 cells (NCCS, Pune, India) transduced to stably express human ACE2 receptor (A549 ACE2), were used for SARS-CoV-2 infection. HEK293T-ACE2, HEK-293T, and A549-ACE2 cells were cultured in complete media prepared using DMEM (12100-038, Gibco) supplemented with 10% HI-FBS (16140-071, Gibco), 100 U/mL Penicillin-Streptomycin (15140122, Gibco) and GlutaMAX™ (35050-061, Gibco). Assessment of cytotoxic effect and the inhibitory potential of identified compounds against SARS-CoV-2 replication was carried out in Vero cells, HEK293T-ACE2, and A549-ACE2. HEK-293T cells (NCCS, Pune) were used for pro-inflammatory cytokine quantification assay and interferon (IFN) induction assay.

The wild type SARSCoV-2/Human/IND/CAD1339/2020 strain was isolated from laboratory confirmed COVID-19 patients in India. After genetic characterization by whole genome sequencing (GenBank accession no: MZ203529), the virus was passaged in Vero cells and supernatant was collected and clarified by centrifugation before being aliquoted for storage at −80 °C. Virus titer was measured in Vero cells by $TCID_{50}$ assay. SARS-CoV-2 isolate Hong Kong/VM20001061/2020 (BEI resources NR-52282), 2020 (hereby referred as SARS-CoV-2) was propagated and titrated in VeroE6 cells. Teneligliptin, Linagliptin, Lithocholic Acid, Vildagliptin, and Flupenthixol (Cayman, India) were dissolved in DMSO and the stock solutions were prepared. The working concentration of drugs prepared in cell culture media was centrifuged at 10,000 rpm for 10 min, and the supernatant was used for all the assays. All procedures related to SARSCoV-2/Human/IND/CAD1339/2020 virus culture and antiviral studies were handled in Biosafety level 3 facility at Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, following standard WHO guidelines. Experiments with SARS-CoV-2 isolate Hong Kong/VM20001061/2020 were performed at the viral biosafety level-3 facility, Indian Institute of Science, Bengaluru, India (Murgolo et al., 2021).

Plasmid constructs

DNA sequence encoding the PLpro of SARS-CoV-2 was codon optimized for bacterial expression (Choudhary et al., 2023). PCR amplification of the gene encoding PLpro was carried out using synthesized gene (Invitrogen, Thermo Fisher scientific) as the template. After restriction digestion, the gene was cloned into the *Nde1* and *Xho1* restriction sites of pET28c bacterial expression vector using 5'-CAGCCATATGGAAGTTCGTACCATTAAAGT TTTTAC-3' forward primer and 5'-GGTGCTCGAGCTATTTGATGGTGGTG-3' reverse

primer. The resulting plasmid was transformed into competent DH5α cells. These transformed cells were grown on Luria–Bertani (LB) agar plates supplemented with 50 μg/mL kanamycin with incubation at 37 °C overnight. Several colonies were picked and grown in 10 mL LB broth containing 50 μg/mL of kanamycin, followed by plasmid isolation. Using the wild type PLpro plasmid as the template, site directed mutagenesis was performed to generate an active site mutant (C111S) by an overlapping PCR strategy, using 5'-AAATGGGCAGATAATAACTCGTATCTGGCAACCG-3' forward primer and 5'-AGTGCGGTTGCCAGATACGAGTTATTATCT-3' as reverse primer. For mammalian expression, gene encoding PLpro was cloned into pcDNA3 vector between *BamH1* and *Xho1* restriction sites using forward primer 5'-ATGAGGATCCATGGAAGTTCGTACCATTAAAGTTTTTACCA-3' and reverse primer 5'-GGTGCTCGAGCTATTTGATGGTGGTG-3'. The plasmids IFN-Beta_pGL3 (Addgene #102597); pRLTK; RIGI 2CARD; plx 304 empty vector (kind gift from Adolfo García-Sastre, Icahn School of Medicine at Mount Sinai, New York) were used for interferon induction experiment. All the clones were verified by DNA sequencing.

Structural comparison of PLpro and USP protein

Pairwise comparisons of secondary structure elements were performed with the web-based Secondary Structure Matching program (SSM), available within the Protein Data Bank (PDBeFold) (Krissinel and Henrick, 2004). For assessing structural similarities, crystal structures of SARS-CoV-2 PLpro (PDB ID: 6W9C), HAUSP/USP7 (PDB ID: 2F1Z), and USP2 (PDB ID: 5XU8) were downloaded from RCSB-PDB (Berman et al., 2000). The structures were fed into the PDBeFold-SSM portal and the hits from each pairwise comparison are ranked by their RMSD values, Q-score, and the number of aligned residues. The 3D alignment file was downloaded and analyzed in PyMOL (Schrodinger LLC, 2015) to observe conserved loops and residues. To gain more insights into the conserved residues at the substrate binding pockets (Ub/ISG15 binding site), multiple sequence alignment of SARS-CoV-2 PLpro (PDB ID: 6W9C), HAUSP/USP7 (PDB ID: 2F1Z), and USP2 (PDB ID: 5XU8) was done using Clustal Omega/ESPript 3.0 (Robert and Gouet, 2014).

Structure-based identification of inhibitors

An *in-house* library of compounds containing previously reported DUBs inhibitors and cyanopyrrolidine ring compounds was prepared, and screened against PLpro to identify potential antivirals against SARS-CoV-2 (Supplementary table 1). The SDF files for three-dimensional structures (3D) of all compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database. 3D-crystal structure of PLpro of SARS-COV-2 (PDB ID: 6W9C) was retrieved from RCSB-PDB. Using the automated function of AutoDock

Vina (Trott and Olson, 2010), refinement of protein was done by addition of polar hydrogen atoms and the protein was saved in PDBQT format for further docking studies.

Before performing structure-based virtual screening, all the ligands were energy minimized by Universal Force Field (UFF) using Open Babel in PyRx 0.8 (Dallakyan and Olson, 2015; O'Boyle et al., 2011). SDF files of all ligands were converted into PDBQT format for virtual screening against PLpro using an in-built Open Babel and AutoDock Vina module of PyRx 0.8. The grid centers for docking search space were set as X= -34.1, Y= 20.3, and Z=33.8, and the dimensions (Å) for X, Y, and Z axis were set as 36.4, 27.8, and 31 respectively. The size of the grid box was set centering on the substrate binding site for PLpro with a default exhaustiveness value of 8 for all ligands. A total of 9 different poses were generated for each ligand. Visual examination of the docked complexes possessing the highest binding affinities was carried out using PyMOL for analyzing hydrogen and hydrophobic interactions. Top-ranked compounds displaying interactions similar to GRL0617 for targeted site of PLpro, possessing high binding affinities, and low RMSD values (< 2 Å) were sorted out. Of these hits, the compounds that were recently reported as SARS-CoV-2 inhibitors in other studies were excluded from further studies.

Molecular docking of the ligands with PLpro of SARS-CoV-2 was carried out at two target sites, the substrate binding cleft near the BL2 groove and the Ub/ISG15 binding site (S2 site), using AutoDock 4.2.6 (Trott and Olson, 2010). Crystal structure of PLpro of SARS-CoV-2 (PDB ID: 6W9C) was downloaded from PDB and the PDBQT file of this energy minimized structure was used. AutoDock and AutoGrid tools available with AutoDock4 were used to calculate grid maps for pre-calculation of interaction energies of all interacting atoms. The center points for substrate-binding cleft were set as X= -34.4, Y= 27.1, and Z= 33.9; grid box dimensions were 100 Å ×100 Å×100 Å. Grid box dimensions for re-docking studies at the S2 Ub/ISG15 binding site were 80 Å ×60 Å×80 Å and grid center points were set as X= -17.6, Y=40.9, and Z= 11.5. Lamarckian genetic algorithm (GA) with a combination of maximum grid-based energy evaluation method was used for docking studies. The program was run for a maximum number of 25 genetic algorithm runs. All other parameters were set as default. A previously reported PLpro inhibitor GRL0617 was used as a reference control (Fu et al., 2021). Out of all the possible poses generated, the pose showing maximum binding affinity, hydrogen, hydrophobic interactions, and lowest RMSD values were chosen for detailed visual analysis using LigPlot and PyMOL.

To gain a deeper understanding of the stability of protein-ligand docked complexes, MD simulation was carried out for the docked complexes of ligands targeting the substrate binding cleft and the S2-binding site, using GROMACS 2022.2 program with a CHARMM force field on Ubuntu-based LINUX workstation. For performing all MD simulations of protein-ligands, the topology files of selected ligands were generated using CHARMM36 General Force Field (CGenFF). The protein-ligand complexes were solvated in a cubic box and the steepest descent algorithm for 50,000 iteration steps was used to perform energy minimization. Two different system equilibration phases were carried out for 500,000 steps. The first phase of system

equilibration was performed at a constant number of particles, volume, and temperature (NVT) at 300K with short range electrostatic cut-off of 1.2 nm. The second phase involved a constant number of particles, pressure, and temperature (NPT) ensemble at 300 K, for each step of 2 fs. Finally, the MD simulation was carried out for 100 ns with integration time frame steps of 2 fs. The RMSD values were calculated for PLpro protein and for all protein-ligand complexes.

PLpro expression, purification, and inhibition assay

For expression in *E. coli*, the plasmid coding for PLpro was transformed into expression host *Rosetta* (DE3; Novagen, USA) and then cultured in 1 L LB broth medium at 37 °C. The expression of PLpro protein was induced using 0.5 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG) along with the addition of 1mM $ZnCl_2$ to log phase culture (OD_{600} = 0.6), and the cells were further grown overnight at 18 °C and 180 rpm in an incubator. Cells were harvested by centrifugation (6000 rpm for 10 min at 4 °C) and were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.5, 500 mM NaCl, and 5mM DTT). Cells were homogenized using French press (Constant Systems Ltd, Daventry, England) and the cellular debris was removed by centrifugation at 10000 rpm for 1.5 h at 4 °C. The clarified supernatant was loaded onto pre-equilibrated nickel-nitrilotriacetic acid (Ni-NTA) (Qiagen, USA) affinity column and flow-through was collected after incubation for 30 min at 4 °C. After washing the resin with binding buffer (50 mM Tris-HCl, pH 8.5, 500 mM NaCl, and 5mM DTT), the recombinant histidine tagged PLpro protein was eluted with 100-500 mM imidazole in binding buffer. The eluted fractions were analyzed using 15% SDS-PAGE. The fractions containing pure PLpro protein were pooled together and dialyzed against buffer containing 20 mM Tris-HCl, pH 8.5, 200 mM NaCl, and 5mM DTT. The dialyzed protein was concentrated using Amicon centrifugal filters (Millipore, Burlington, MA, USA) with a molecular weight cutoff of 10 kDa. The concentration of purified PLpro was determined by using the Bradford assay. Expression and purification of C111S inactive PLpro mutant was performed using the same protocol as described for the wild type PLpro.

To characterize *in vitro* enzymatic activity of SARS-CoV-2 PLpro and to assess the inhibitory potential of identified compounds, a high-throughput FRET based enzymatic assay was developed as described previously (Choudhary et al., 2023; Shan et al., 2021). To carry this out, a commercially available fluorogenic substrate Z-Arg-Leu-Arg-Gly-Gly-7-amido-4-methylcoumarin (Z-RLRGG-AMC) (Biolinkk, India) representing the C-terminal residues of ubiquitin was used. The experiment was carried out in 96-well non-binding black plates (Corning) in reaction buffer containing 50 mM HEPES (pH 7.5). The protease diluted in reaction buffer was added to each well at a final concentration of 1 μM. Enzymatic reaction was initiated with the addition of fluorogenic substrate peptide at a concentration of 1.5 μM and the fluorescence signals were monitored continuously using a plate reader (Synergy HT, BioTek Instruments, Inc) with filters for excitation at 360/40 nm and emission at 460/40 nm for 30 min. PLpro C111S was used as a negative control to determine whether signal generation was

dependent on proteolytic activity of enzyme. Data from duplicate set of readings was averaged together to plot a graph for relative fluorescence and time.

To assess the inhibitory potential of screened compounds, the assay was assembled as follows: 1 μM PLpro in 50 mM HEPES buffer was incubated with increased concentration of compounds (Cayman chemicals, USA) ranging from 0.1 μM to 500 μM and was dispensed into wells of 96-well plate. Reactions were initiated with 1.5 μM of fluorogenic substrate peptide and the fluorescence signals were monitored continuously for 10 min at excitation wavelength of 360/40 nm and emission wavelength of 460/40 nm. Data from duplicate set of readings were averaged together and a graph for percentage inhibition vs concentration was plotted for each selected compound after non-linear curve fitting using GraphPad Prism. Concentration of inhibitor that resulted in IC_{50} was calculated.

Binding kinetic analysis using Surface Plasmon Resonance

The binding kinetics and affinity of identified compounds to PLpro of SARS-CoV-2 were analyzed by SPR using a Biacore T200 system (GE Healthcare) at 25 °C. In brief, histidine-tagged PLpro was diluted to 10 μM concentration at pH 7.3 and was then captured on NTA chip in 1X Phosphate buffer saline (1X PBS) running buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) degassed and filtered through 0.22 μm membrane filter. The binding kinetic studies were performed by collecting the data at 25 °C by injecting the concentration series of analyte over the surface of chip having the immobilized PLpro with the following injection parameters: 10μL/min flow rate and 60 s association and dissociation phases. The surface was regenerated after each dissociation phase. Equilibrium dissociation constants (K_D) of each pair of PLpro-ligand interaction were calculated using Biacore Evaluation Software (GE Healthcare) by fitting to a 1:1 Langmuir binding model.

Viral growth and cytotoxicity assays in the presence of inhibitors

To determine the cytotoxic profiles of identified compounds on Vero cells, HEK293T-ACE2, and A549-ACE2, MTT assay was performed as described previously (Mudgal et al., 2022). Briefly, cells were seeded onto wells of 96-well cell-culture plate at a density of 1×10^4 cells/well and the plates were incubated at 37 °C and 5% CO_2 overnight for proper adherence of cells. The culture media was removed and the monolayer of cells was treated with increased dilutions of compounds (1-100 μM) prepared in DMEM media in triplicates. Post incubation period of 48 h, the media was removed, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution (5 mg/mL) was added and incubated for 4 h at 37 °C in a humidified incubator with 5% CO_2. Following this, the MTT solution was removed and the formazan crystals were dissolved in DMSO. Absorbance was measured at a wavelength of 550 nm using a multimode plate reader (Synergy HT, BioTek Instruments, Inc). Results were expressed as percentage viability of cells in comparison to untreated cell control. Percentage viability for each compound was calculated and a dose-response curve was plotted using GraphPad Prism software.

Viral load reduction assay was performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Briefly, Vero cells were seeded and cultured in 24-well plate in DMEM media supplemented with 10% FBS and 100 units of penicillin and 100 mg streptomycin/mL, at 37 °C and 5% CO_2. 3 h before infection, the cells were washed with PBS and the media was replaced with DMEM (2% FBS) containing gradient of diluted experimental compounds. Post incubation, the cells were washed with PBS and were infected with SARS-CoV-2 at a Multiplicity of infection (MOI) of 0.01 in media without FBS and again incubated for 1.5 h. After incubation, the viral inoculum was removed and the cells were washed with PBS and then replenished with fresh medium containing dilutions of compounds. Plates were then incubated for 48 h at 37 °C and 5% CO_2. At 48 hours post infection (hpi) the plates were freezed at -80ºC. On the following day plate was thawed, and viral RNA was extracted from cell lysate of SARS-CoV-2 infected cells using HiPurA™ Viral RNA Purification Kit, according to the manufacturer's instructions. One-step qRT-PCR was performed with primers targeting the SARS-CoV-2 envelope (E-gene) and RdRp region using the commercially available COVISure-COVID-19 Real-Time PCR kit (Genetix), as per the manufacturer's instructions, to quantify the virus yield. The thermocycling conditions consist of 15 min at 50 °C for reverse transcription, 95 °C for 2 min, followed by 40 cycles of 30 s at 95 °C and 30 s at 60 °C. Azithromycin was used as positive control (data not shown). All experiments were performed in duplicates. Percentage inhibition was calculated based on ΔΔCt. The percentage inhibition versus concentration graph was plotted to calculate the EC_{50} values using the linear regression.

Infectious virus production by compound treated and infected cells as compared to only infected cells was also evaluated by the standard 50% tissue culture infectious dose ($TCID_{50}$) and was expressed as $TCID_{50}$/mL (Reed and Muench, 1938). For this purpose, 4×10^4 Vero cells/well were seeded in a 96-well plate and the plate was incubated at 37 °C and 5 % CO_2, till confluency. The freeze-thawed cell lysate of cells infected and treated with different concentrations of compounds collected at 48 hpi of the antiviral assay was subjected to 10-fold serial dilutions (10^1 to 10^8). Compound untreated and infected samples were used as positive control, and samples with only cell-culture media were used as a negative control. These dilutions were inoculated on Vero cells. Cells were incubated for 3 to 4 days at 37 °C and 5 % CO_2. The plate was monitored daily for the appearance of a cytopathic effect (CPE). Post incubation, the viral inoculum was removed, and 10% formaldehyde (in PBS) was used to fix cells for 2 h at room temperature, followed by staining with 0.5% crystal violet solution for 1 h. Percent infected dilutions immediately above and immediately below 50% were determined. $TCID_{50}$ was calculated on the basis of Reed and Muench method (Reed and Muench, 1938).

Validation of antiviral activity in human cell lines

HEK293T-ACE2 and A549-ACE2 cells were seeded in 24 well plates and used for antiviral assay the next day. Single non-toxic concentrations of Teneligliptin, Linagliptin, Lithocholic Acid or Flupenthixol were prepared in infection medium (DMEM containing 2% FBS) and used to pre-treat cells for 3 h. For Linagliptin and Lithocholic acid, cells were treated with increasing

doses of the drugs to estimate IC_{50}. Cells were then washed with warm PBS and infected with 0.01 MOI SARS-CoV-2 in DMEM without FBS for 2 h. The inoculum was then removed, cells washed with PBS and incubated with infection media containing the drug concentrations. After 48 (HEK293T ACE2) or 72 h (A549 ACE2) total RNA was extracted using TRIzol (Thermo Fisher, 15596018) as per manufacturer's instructions, and viral copy number was estimated by qRT PCR.

qRT-PCR: Equal amount of RNA was used to determine viral load using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, AM1005). The following primers and probes targeting the SARS-CoV-2 N-1 gene were used for amplification. Forward primer: 5'GACCCCAAAATCAGCGAAAT3' and Reverse primer: 5' TCTGGTTACTGCCAGTTGAATCTG3', Probe: (6-FAM / BHQ-1) ACCCCGCATTACGTTTGGTGGACC. The Ct values were used to determine viral copy numbers by generating a standard curve using the SARS-CoV-2 genomic RNA standard. All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad Software, USA). In all cases, a p-value < 0.05 is considered significant.

Pro-inflammatory cytokine quantification and Interferon induction assay

24 h prior to transfection, HEK293T cells were seeded in the wells of 24-well plate. The media was removed and the cells were stimulated with 1 µg/mL polyinosinic:polycytidylic acid (poly I:C; Sigma) using Lipofectamine 2000 (Invitrogen). Post incubation period of 6 h, the media over the cells was removed, fresh OptiMEM (Gibco) containing the compounds was added to cells (Devaraj et al., 2007; Thorne et al., 2022). 24 h post transfection, total RNA isolation was done using TRIzol (RNAiso; Takara) by following manufacturer's instruction and was reverse-transcribed into first-strand cDNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio) in accordance with manufacturer's protocol. For determination of effects of compounds on interferon, 500 ng of cloned pcDNA3 vector encoding PLpro protein of SARS-CoV-2 was transfected in HEK293T cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Cells were stimulated 24 h after plasmid transfection with 1 µg/mL poly I:C (Sigma) using Lipofectamine 2000 (Invitrogen). After an incubation of 6 h, fresh OptiMEM media containing the compounds was added to cells. Mock treated cells or cells transfected with 500 ng of empty pcDNA3 vector or were included as controls (Devaraj et al., 2007; Shin et al., 2020; Thorne et al., 2022). RNA was isolated and was used for CDNA synthesis using PrimeScript 1st strand cDNA Synthesis Kit (Takara; India). The cDNA was used for Gene expression analyses for IL6, TNFα, and IFN-β cytokines, using KAPA SYBR fast universal qPCR kit (Sigma-Aldrich, USA) on QuantStudio real-time PCR system (Applied Biosystems, IN) with the expression of β-actin as the internal control. Based on the ΔΔCt method, The fold change in infected cells compared to corresponding controls was calculated after normalizing to the β-actin gene. All reactions were carried duplicates in a 96-well plate (Applied Biosystems, IN). Fold change was calculated by taking cells treated with poly(I:C) as 1. The primer sequences used for qRT-PCR are listed in Supplementary Table 3.

For interferon induction assay, HEK-293T cells were co-transfected with 50 ng IFNβ-luc firefly luciferase reporter plasmid, 20 ng RIGI 2CARD and 20 ng of pRL-TK Renilla luciferase reporter plasmid, along with/without 500 ng of plasmid expressing SARS-CoV-2 PLpro or empty vector. Transfection was done using Lipofectamine™ 2000 Transfection Reagent (11668019, Invitrogen) as per manufacturer's instructions. 3 h post transfection, the media was replaced with complete medium containing 50 µM Teneligliptin, 50 µM Linagliptin, 25 µM Lithocholic Acid or 10 µM Flupenthixol. Cells were incubated for 24 h post transfection, then harvested in passive lysis buffer (E1941, Promega) and luciferase expression quantified using a dual luciferase assay (E1980, Promega). All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad Software, USA). In all cases, a p-value < 0.05 is considered significant.

Crystallization and structure determination

Purified SARS-CoV-2 PLpro, in the inactive mutant form (C111S), was crystallized using sitting-drop vapor diffusion method. To achieve this, the purified protein was concentrated up to 15 mg/mL and the crystal trays were set up at various protein-to-precipitant ratios (1:1, 2:1, and 1:2) with reservoir buffer containing crystallization condition [0.1 M Tris-HCl (pH 7.5), 1 M NaH_2PO_4, 1.2 M K_2HPO_4, and 10% Glycerol] in 96-well sitting-drop plate (Corning, USA). Diffraction quality crystals were produced after 1 week at 4 °C. The crystals were manually harvested from the drop and were cryo-protected by reservoir well solution plus 10% glycerol and snap-frozen in liquid nitrogen for diffraction data collection. For co-crystallization of PLproC111S-Lithocholic acid complex, a 10-fold molar excess of the inhibitor was added to PLproC111S (15 mg/mL) and was incubated overnight at 4 °C. The sample for complex was then clarified by centrifugation at 10,000 rpm for 20 min followed by setting up of crystal tray using sitting drop vapor diffusion method at protein-to-precipitant ratio of 1:1 using crystallization condition similar to that for apo PLproC111S [0.1 M Tris-HCl (pH 7.5), 1 M NaH_2PO_4, 1.2 M K_2HPO_4 and 10% Glycerol]. To obtain Linagliptin soaked PLpro crystals, apo crystals for PLproC111S were transferred into crystallization solution supplemented with Linagliptin (10 mM). The crystals were allowed to soak, undisturbed for 2-6 h at 4 °C and the soaked crystals were then cryoprotected in reservoir liquor supplemented with 10% glycerol.

X-ray diffraction data for PLproC111S apo and its complex with identified compounds was collected at the Home Source, Macromolecular crystallography Unit, IIC, IIT Roorkee. Datasets were processed using Aimless module of CCP4i2 and the structures were determined by molecular replacement (also a part of the CCP4i2 package) with a SARS-CoV-2 PLpro structure (PDB 6WRH) as a search model. Models for SARS-CoV-2 PLproC111S and its complex with selected compounds were subjected to sequential rounds of model building using COOT and refinement of atomic coordinates and B-factors in refmac5 that allowed for the correct placement of sidechains and loops. All structural analysis and figure preparation was carried out by

software PyMOL (Schrödinger, New York, NY, USA). Models were validated with MolProbity. The parameters for X-ray data collection and refinement statistics are presented in **Table 2**.

HPLC

High-Performance Liquid Chromatography (HPLC) analysis for inhibitor was carried out on a Waters HPLC system equipped with Sunfire C18 reverse-phase column (250 × 4.6 mm, 5 µm particle size), 1525 binary pump and 2998 photodiode array detector (PDA). The degassed and vacuum filtered mobile phase consisted of water containing 0.1% Orthophosphoric acid pH 4.3 (A) and methanol (B) which were employed in the isocratic flow of 9A: 41B at a flow rate of 0.5 mL/min. Injection volume was set 20 µL and the column temperature was 28 °C. The ligand was monitored at 230 nm. Sample peaks were identified by matching retention time and UV spectrum with authentic standards.

Animals and dosing

A total of 50 female mice, aged 6–8 weeks and weighing 18–20 g, were housed in a Biosafety Level-3 viral laboratory with unlimited access to feed and water. Linagliptin and Molnupiravir were dissolved in 0.5% carboxymethyl cellulose (CMC) in PBS. Mice in the respective groups were either gavaged orally or injected intraperitoneally with 200 µL of the solution, starting 6 hours post-infection and continuing until sacrifice. The dosing regimen was 30 mg/kg or 60 mg/kg body weight (b.w.) for Linagliptin, and 100 mg/kg or 200 mg/kg b.w. for Molnupiravir. Control groups, both infected and uninfected, received 200 µL of 0.5% CMC alone (**Figure 8**).

Virus infection and animal sacrifice

The animals were anesthetized via intraperitoneal injection of ketamine (150 mg/kg, Bharat Parenterals Limited) and xylazine (10 mg/kg, Indian Immunologicals Ltd.), then intranasally challenged with 10^5 plaque-forming units (PFU) of mouse-adapted SARS-CoV-2 (MA10) in 100 µL of PBS. Body weight was recorded daily, and the animals were sacrificed on day 4 post-infection using an overdose of xylazine. Lung samples were collected for virological analysis (right lobe) and histopathological examination (left lobe).

Quantification of lung viral load by qRT PCR

Lung samples from each mouse were homogenized using a stainless-steel bead-based homogenizer, and total RNA was isolated following the manufacturer's instructions for TRIzol (15596018, Thermo Fisher). Viral RNA was quantified using a 10µL reaction mixture containing 100ng of RNA per sample in a 384-well block, with the AgPath-ID™ One-Step RT-PCR kit (AM1005, Applied Biosystems). Primers and probes targeting the SARS-CoV-2 N-1 gene were used, with the following sequences: Forward primer: 5' GACCCCAAAATCAGCGAAAT 3', Reverse primer: 5' TCTGGTTACTGCCAGTTGAATCTG 3', and Probe: (6-FAM / BHQ-1)

ACCCCGCATTACGTTTGGTGGACC. Ct values were used to determine viral copy numbers by generating a standard curve from a SARS-CoV-2 genomic RNA standard.

Lung histology

Lung tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin blocks. Tissue sections of 5 μm thickness were stained with Hematoxylin and Eosin (H&E). Slides were examined under light microscopy for three histological criteria in the lung (Alveolar infiltration and exudation, vasculature inflammation and peribronchiolar infiltration with epithelial desquamation) and were scored on a scale of 1 to 3 (mild=1, moderate=2, severe=3).

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Data and materials availability

All data are available in the main text or the supplementary materials. X-ray coordinates and structure factors are deposited at the RCSB Protein Data Bank under accession codes 8XTD and 8X1X.

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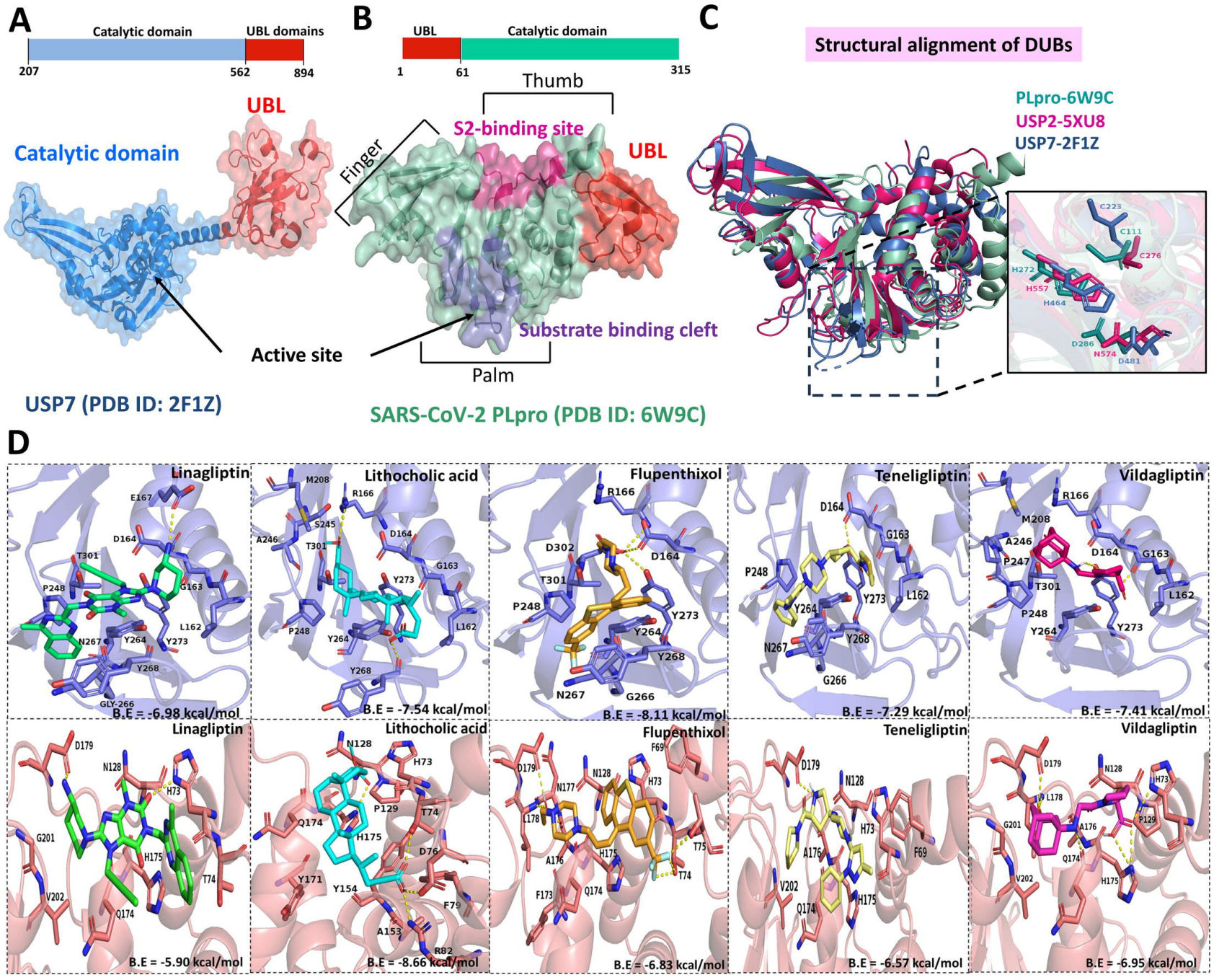
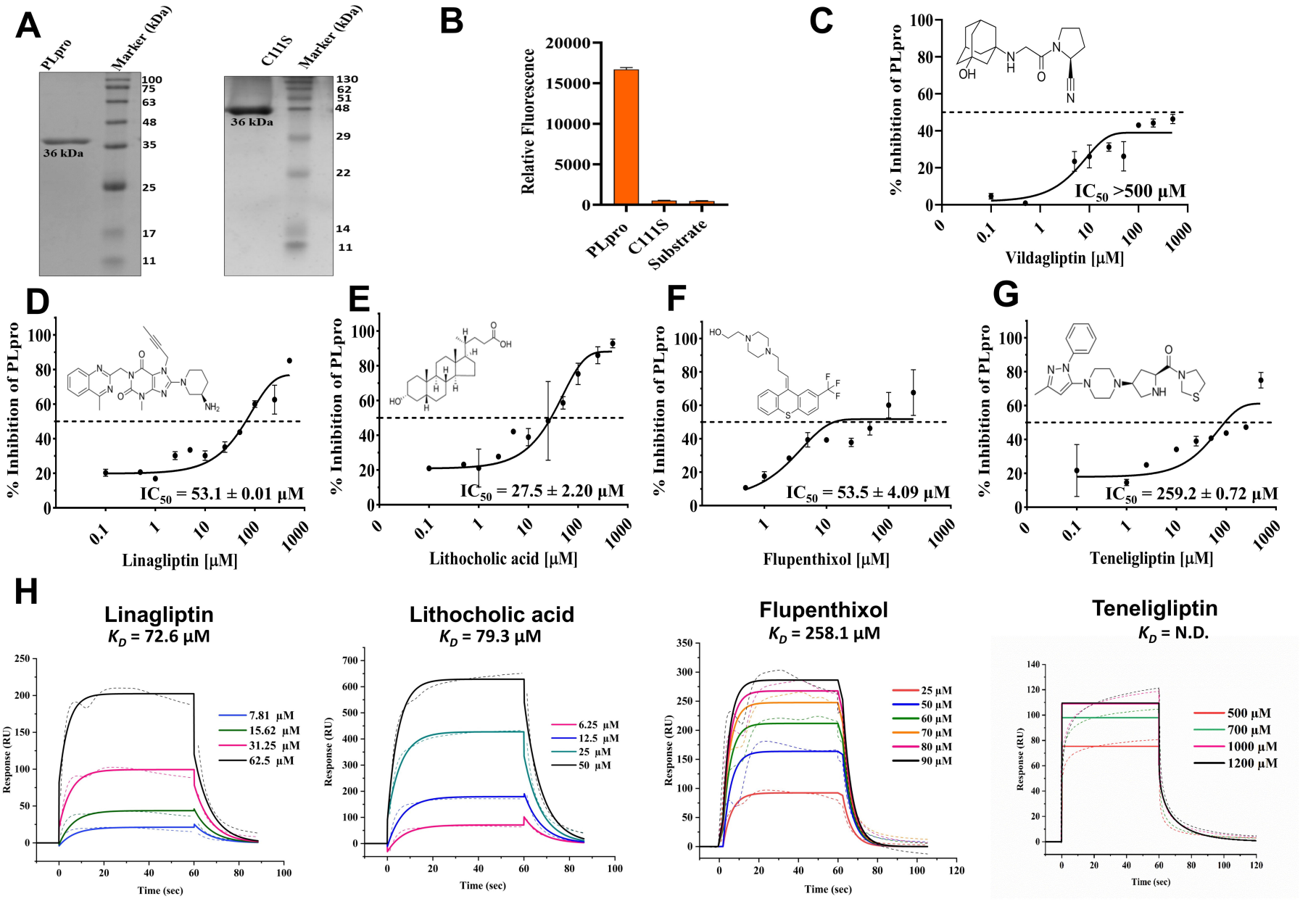
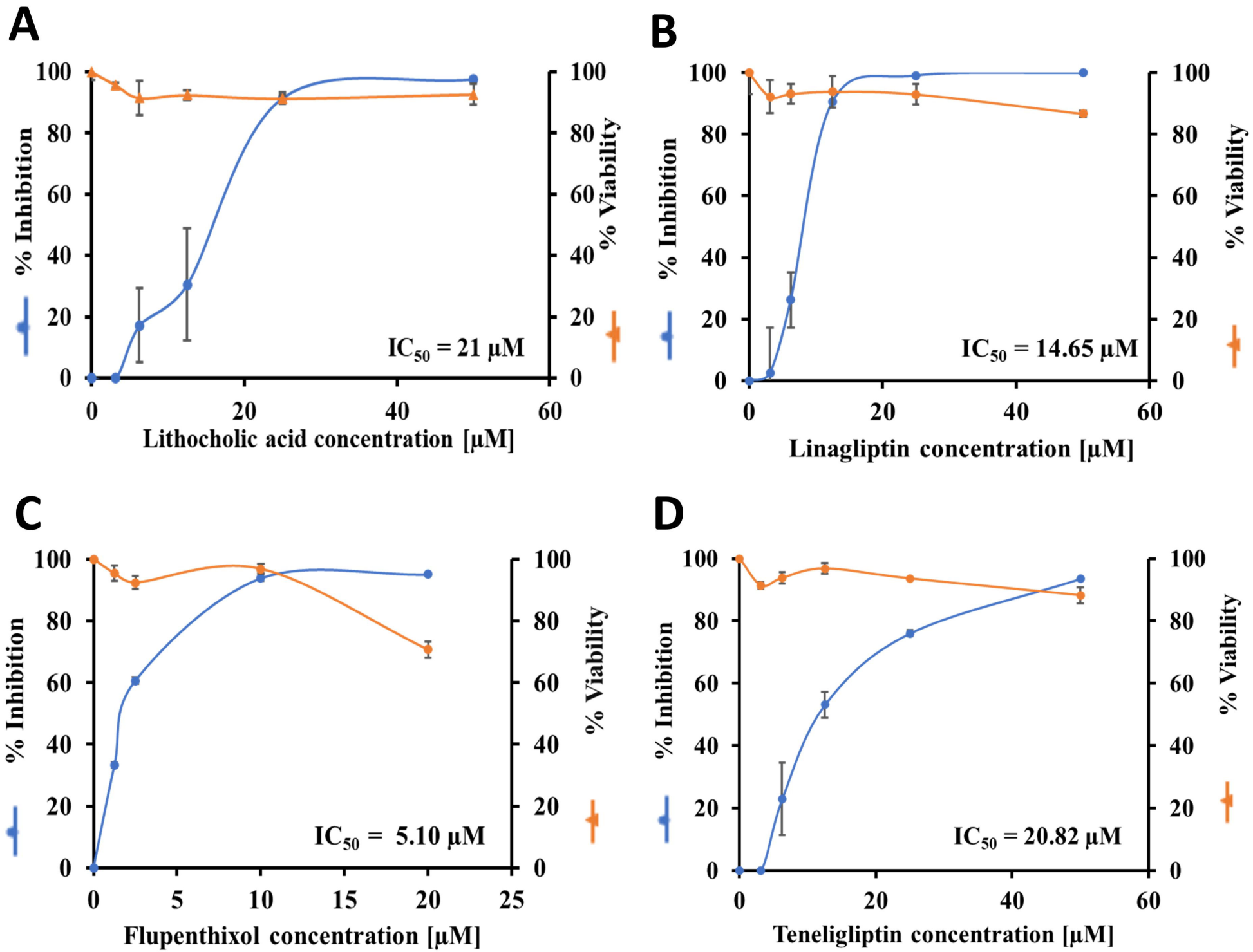
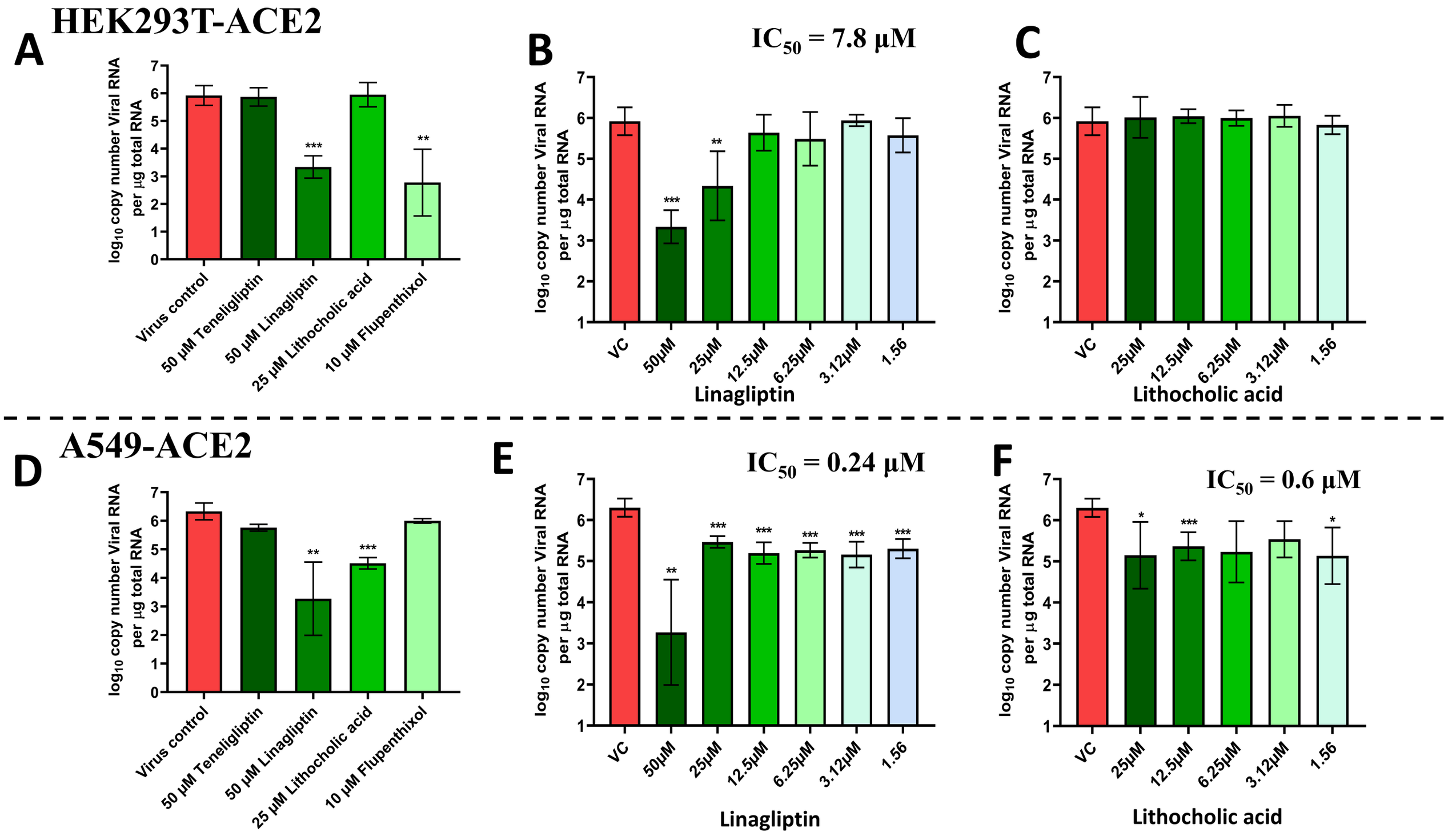
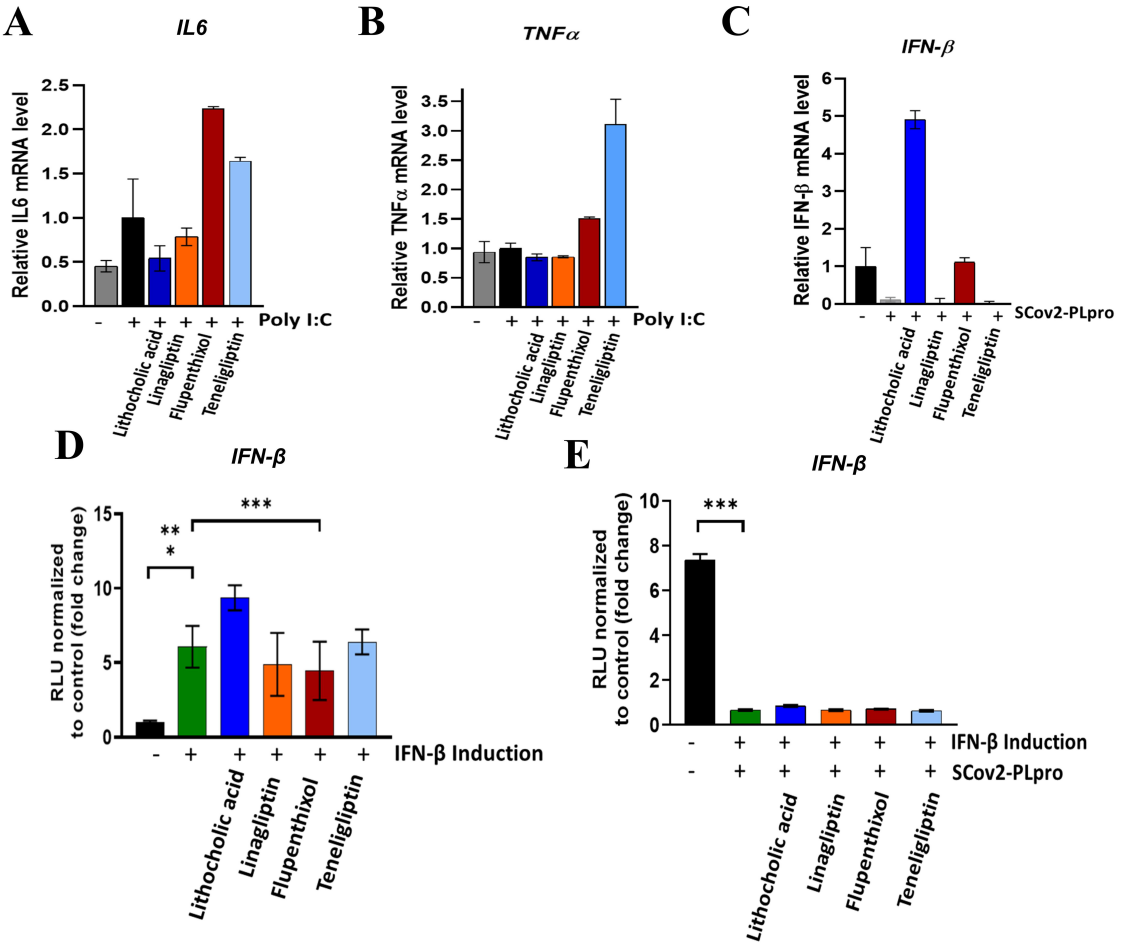
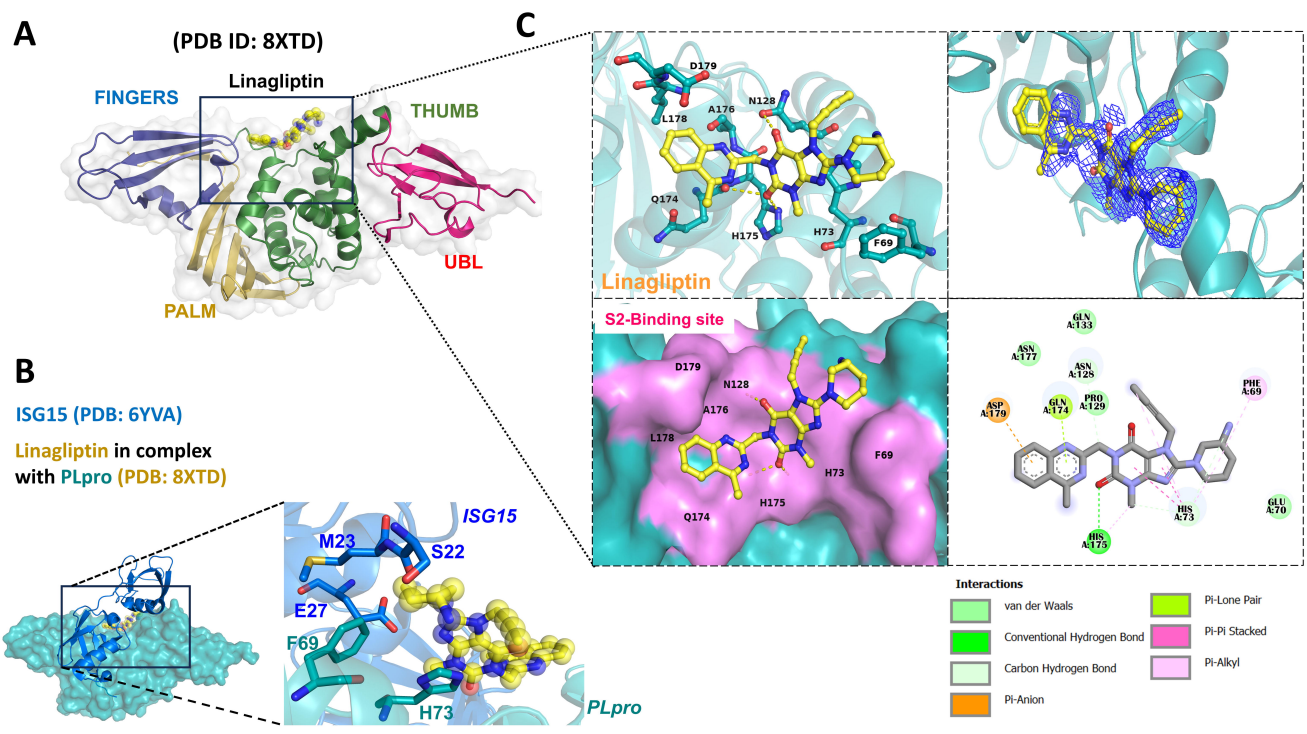

A
Catalytic domain
UBL domains
207
562
894
UBL
Catalytic domain
Active site
USP7 (PDB ID: 2F1Z)
B
UBL
Catalytic domain
1
61
315
Thumb
S2-binding site
UBL
Finger
Substrate binding cleft
Palm
SARS-CoV-2 PLpro (PDB ID: 6W9C)
C
Structural alignment of DUBs
PLpro-6W9C
USP2-5XU8
USP7-2F1Z
C223
C111
C276
H272
H557
H464
D286
N574
D481
D
Linagliptin
B.E = -6.98 kcal/mol
Lithocholic acid
B.E = -7.54 kcal/mol
Flupenthixol
B.E = -8.11 kcal/mol
Teneligliptin
B.E = -7.29 kcal/mol
Vildagliptin
B.E = -7.41 kcal/mol
Linagliptin
B.E = -5.90 kcal/mol
Lithocholic acid
B.E = -8.66 kcal/mol
Flupenthixol
B.E = -6.83 kcal/mol
Teneligliptin
B.E = -6.57 kcal/mol
Vildagliptin
B.E = -6.95 kcal/mol

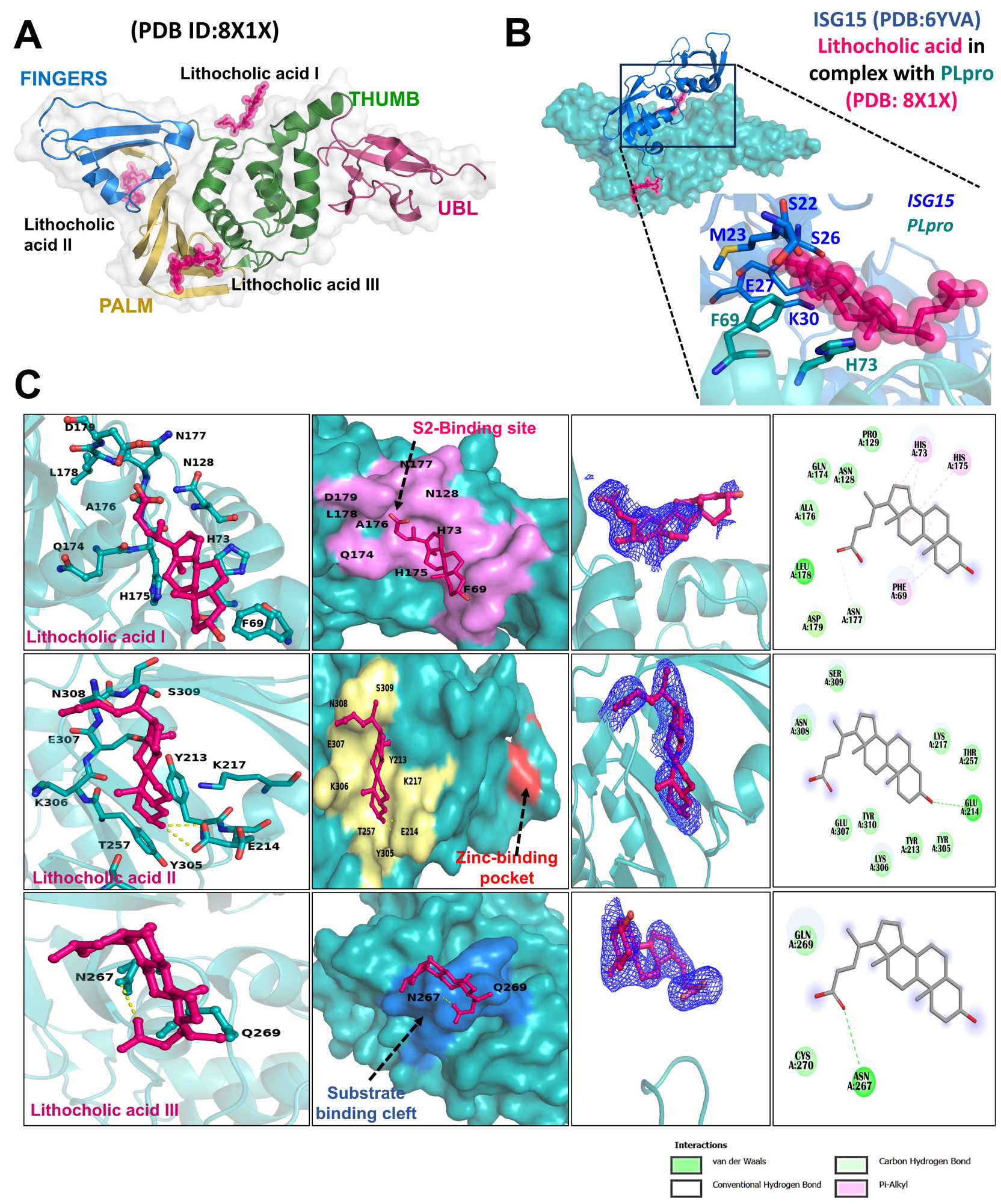

A
PLpro
Marker (kDa)
100
75
63
48
35
25
17
11
36 kDa
C111S
Marker (kDa)
130
62
51
48
29
22
14
11
36 kDa
B
Relative Fluorescence
20000
15000
10000
5000
0
PLpro
C111S
Substrate
C
% Inhibition of PLpro
IC50 >500 μM
Vildagliptin [μM]
D
% Inhibition of PLpro
IC50 = 53.1 ± 0.01 μM
Linagliptin [μM]
E
% Inhibition of PLpro
IC50 = 27.5 ± 2.20 μM
Lithocholic acid [μM]
F
% Inhibition of PLpro
IC50 = 53.5 ± 4.09 μM
Flupenthixol [μM]
G
% Inhibition of PLpro
IC50 = 259.2 ± 0.72 μM
Teneligliptin [μM]
H
Linagliptin
KD = 72.6 μM
7.81 μM
15.62 μM
31.25 μM
62.5 μM
Response (RU)
Time (sec)
Lithocholic acid
KD = 79.3 μM
6.25 μM
12.5 μM
25 μM
50 μM
Response (RU)
Time (sec)
Flupenthixol
KD = 258.1 μM
25 μM
50 μM
60 μM
70 μM
80 μM
90 μM
Response (RU)
Time (sec)
Teneligliptin
KD = N.D.
500 μM
700 μM
1000 μM
1200 μM
Response (RU)
Time (sec)

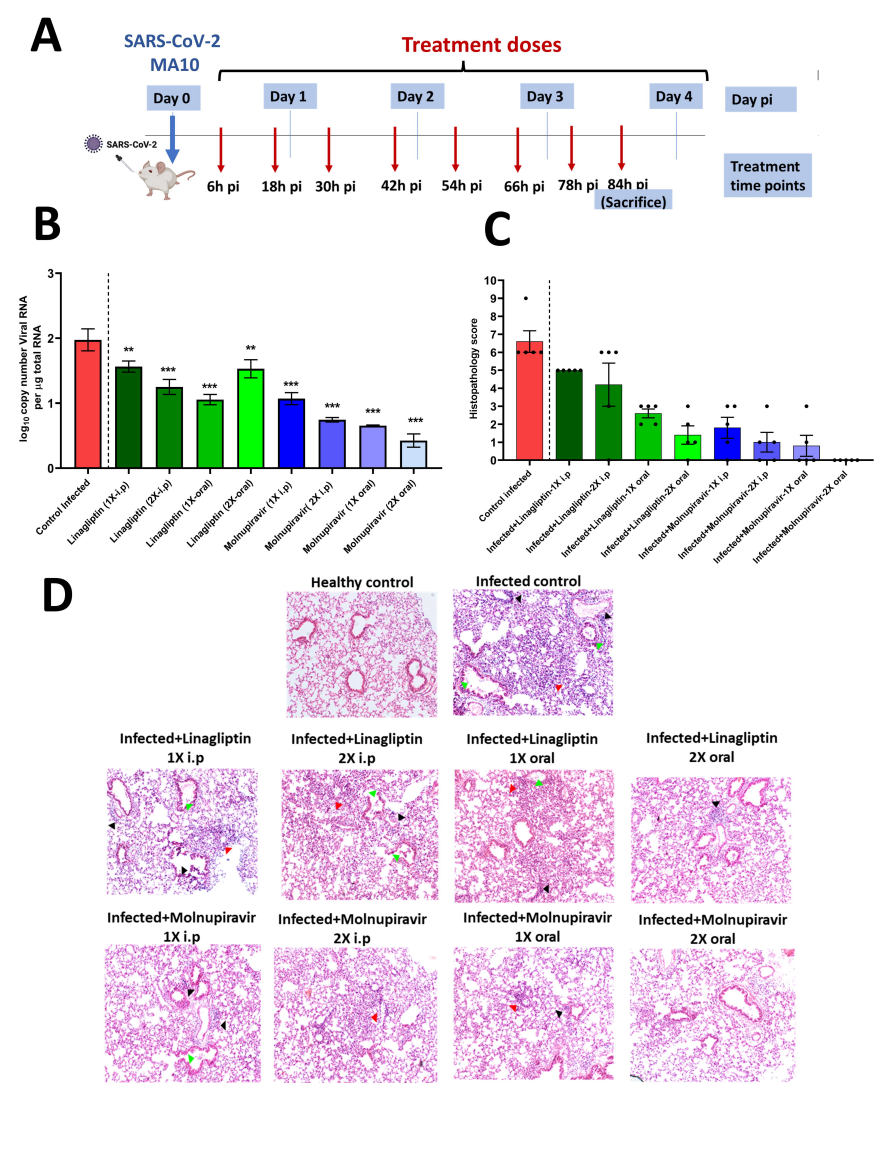

A
% Inhibition
% Viability
IC50 = 21 µM
Lithocholic acid concentration [µM]
B
% Inhibition
% Viability
IC50 = 14.65 µM
Linagliptin concentration [µM]
C
% Inhibition
% Viability
IC50 = 5.10 µM
Flupenthixol concentration [µM]
D
% Inhibition
% Viability
IC50 = 20.82 µM
Teneligliptin concentration [µM]


HEK293T-ACE2
A
log10 copy number Viral RNA per μg total RNA
Virus control
50 μM Teneligliptin
50 μM Linagliptin
25 μM Lithocholic acid
10 μM Flupenthixol
B
IC50 = 7.8 μM
VC
50μM
25μM
12.5μM
6.25μM
3.12μM
1.56
Linagliptin
C
Lithocholic acid
A549-ACE2
D
E
IC50 = 0.24 μM
F
IC50 = 0.6 μM


A
IL6
Relative IL6 mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
- + + + + +
Poly I:C
Lithocholic acid
Linagliptin
Flupenthixol
Teneligliptin
B
TNFα
Relative TNFα mRNA level
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
- + + + + +
Poly I:C
Lithocholic acid
Linagliptin
Flupenthixol
Teneligliptin
C
IFN-β
Relative IFN-β mRNA level
6
5
4
3
2
1
0
- + + + + +
SCov2-PLpro
Lithocholic acid
Linagliptin
Flupenthixol
Teneligliptin
D
IFN-β
RLU normalized to control (fold change)
15
10
5
0
**
*
***
- + + + + +
IFN-β Induction
Lithocholic acid
Linagliptin
Flupenthixol
Teneligliptin
E
IFN-β
RLU normalized to control (fold change)
10
8
6
4
2
0
***
- + + + + +
IFN-β Induction
- + + + + +
SCov2-PLpro
Lithocholic acid
Linagliptin
Flupenthixol
Teneligliptin


A
(PDB ID: 8XTD)
Linagliptin
FINGERS
THUMB
UBL
PALM
B
ISG15 (PDB: 6YVA)
Linagliptin in complex
with PLpro (PDB: 8XTD)
ISG15
M23
S22
E27
F69
H73
PLpro
C
D179
N128
L178
A176
Q174
H175
H73
F69
Linagliptin
S2-Binding site
GLN A:133
ASN A:177
ASN A:128
PHE A:69
ASP A:179
GLN A:174
PRO A:129
GLU A:70
HIS A:73
HIS A:175
Interactions
van der Waals
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Pi-Anion
Pi-Lone Pair
Pi-Pi Stacked
Pi-Alkyl


A
(PDB ID:8X1X)
FINGERS
Lithocholic acid I
THUMB
UBL
Lithocholic acid II
Lithocholic acid III
PALM
B
ISG15 (PDB:6YVA)
Lithocholic acid in complex with PLpro
(PDB: 8X1X)
ISG15
PLpro
S22
M23
S26
E27
F69
K30
H73
C
D179
N177
L178
N128
A176
Q174
H73
H175
F69
Lithocholic acid I
S2-Binding site
N308
S309
E307
Y213
K217
K306
T257
E214
Y305
Lithocholic acid II
Zinc-binding pocket
N267
Q269
Lithocholic acid III
Substrate binding cleft
PRO A:129
HIS A:73
HIS A:175
GLN A:174
ASN A:128
ALA A:176
LEU A:178
PHE A:69
ASP A:179
ASN A:177
SER A:309
ASN A:308
LYS A:217
THR A:257
GLU A:214
GLU A:307
TYR A:310
TYR A:213
TYR A:305
LYS A:306
GLN A:269
CYS A:270
ASN A:267
Interactions
van der Waals
Carbon Hydrogen Bond
Conventional Hydrogen Bond
Pi-Alkyl


A
SARS-CoV-2
MA10
Treatment doses
Day 0
Day 1
Day 2
Day 3
Day 4
Day pi
SARS-CoV-2
6h pi
18h pi
30h pi
42h pi
54h pi
66h pi
78h pi
84h pi
(Sacrifice)
Treatment time points
B
log10 copy number Viral RNA per µg total RNA
Control Infected
Linagliptin (1X i.p)
Linagliptin (2X i.p)
Linagliptin (1X oral)
Linagliptin (2X oral)
Molnupiravir (1X i.p)
Molnupiravir (2X i.p)
Molnupiravir (1X oral)
Molnupiravir (2X oral)
C
Histopathology score
Control infected
Infected+Linagliptin-1X i.p
Infected+Linagliptin-2X i.p
Infected+Linagliptin-1X oral
Infected+Linagliptin-2X oral
Infected+Molnupiravir-1X i.p
Infected+Molnupiravir-2X i.p
Infected+Molnupiravir-1X oral
Infected+Molnupiravir-2X oral
D
Healthy control
Infected control
Infected+Linagliptin 1X i.p
Infected+Linagliptin 2X i.p
Infected+Linagliptin 1X oral
Infected+Linagliptin 2X oral
Infected+Molnupiravir 1X i.p
Infected+Molnupiravir 2X i.p
Infected+Molnupiravir 1X oral
Infected+Molnupiravir 2X oral