Losartan Inhibits SARS-CoV-2 Replication *in vitro*

Reza Nejat[1, 2], Ahmad Shahir Sadr[3,4], Brendan T. Freitas[5], Jackelyn Murray[6], Scott D. Pegan[5], Ralph A. Tripp[6], David J. Najafi[7]

[1]Department of Anesthesiology and Critical Care Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [2]ICU Chief, Bazarganan Hospital, Tehran, Iran; [3]Bioinformatics Research Center, Cheragh Medical Institute and Hospital, Kabul, Afghanistan; [4]Department of Computer and Data Sciences, Faculty of Mathematical Sciences, Shahid Beheshti University, Tehran, Iran; [5]Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, Georgia 30602; [6]Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia , USA; [7]Alliance Retinal Consultants, La Mesa California, USA

Corresponding author: David J. Najafi, Orcid ID: 0000-0001-5270-8922, Alliance Retina Consultants, 8262 University Ave, La Mesa, CA, 91942, USA; TEL: (619) 668-0045; email: dnajafimd@gmail.com

ABBREVIATION. ACE2: Angiotensin-Converting Enzyme II; ARB: angiotensin receptor blockers; AT1R: Angiotensin II type 1 receptor; AT2R: Angiotensin II type 2 receptor; DUB: deubiquitinase; PLpro: papain-like protease; proISG15: precursor ISG15; RAS: renin-angiotensin system; RBD: receptor binding domain; TLR: Toll-like receptor; Ub: ubiquitin; poly Ub: polyubiquitin

ABSTRACT - Purpose: SARS-CoV-2 infection is associated with substantial mortality and high morbidity. This study tested the effect of angiotensin II type I receptor blocker, losartan, on SARS-CoV-2 replication and inhibition of the papain-like protease of the virus. **Methods:** The dose-dependent inhibitory effect of losartan, in concentrations from 1μM to 100μM as determined by quantitative cell analysis combining fluorescence microscopy, image processing, and cellular measurements (Cellomics analysis) on SARS-CoV-2 replication was investigated in Vero E6 cells. The impact of losartan on deubiquitination and deISGylation of SARS-CoV-2 papain-like protease (PLpro) were also evaluated. **Results:** Losartan reduced PLpro cleavage of tetraUbiquitin to diUbiquitin. It was less effective in inhibiting PLpro's cleavage of ISG15-AMC than Ubiquitin-AMC. To determine if losartan inhibited SARS-CoV-2 replication, losartan treatment of SARS-CoV-2 infected Vero E6 was examined. Losartan treatment one hour prior to SARS-CoV-2 infection reduced levels of SARS-CoV-2 nuclear protein, an indicator of virus replication, by 80% and treatment one-hour post-infection decreased viral replication by 70%. **Conclusion:** Losartan was not an effective inhibitor of deubiquitinase or deISGylase activity of the PLpro but affected the SARS-CoV-2 replication of Vero E6 cells *in vitro*. As losartan has a favorable safety profile and is currently available it has features necessary for efficacious drug repurposing and treatment of COVID-19.

INTRODUCTION

COVID-19 pandemic caused by SARS-CoV-2 has created a global health and economic crisis. SARS-CoV-2 is an enveloped non-segmented single-stranded positive sense RNA virus belonging to the Coronaviridae family which includes SARS-CoV-1 and Middle East Respiratory Syndrome (MERS) coronavirus [1, 2]. SARS-CoV-2 has 79% homology with SARS-CoV-1 at the genomic level [1-4].

COVID-19 is a multi-organ disease involving the lungs, cardiovascular system, gastrointestinal tract, and the central nervous system [5]. There is currently no effective antiviral drug, and the recently approved vaccines are not expected to be generally available globally [6]. Drug repurposing is a

potential alternative strategy to restrict the disease. A drug that is globally available, safe, with beneficial properties in ameliorating the pathological changes of the disease and with minimum side-effects could drastically impact the management of this pandemic. SARS-CoV-2, like SARS-CoV-1, uses Angiotensin Converting Enzyme 2 (ACE2) to enter the host cells [7]. Attachment of the receptor binding domain (RBD) of S1 subunit of the spike protein (S protein) to ACE2 mediates the cell entry of the virus [8]. Upon SARS-CoV-2 entry into the host cell, replication is initiated through translation of two open reading frames, ORF1a and ORF1b, which produces two replicase polyproteins (pp1a/pp1ab) containing non-structural proteins (nsps) contributing to its replication [9, 10]. Autoproteolytic

cleavage of pp1a/pp1ab by cysteine proteases of the virus, papain-like protease (PLpro) and 3Chemotrypsin-like proteinase (3CLpro or Mpro), releases the nsps [11, 12]. Having 83% homology with SARS-CoV-1 PLpro, this cysteine protease of SARS-CoV-2 has the ability to alter the intracellular microenvironment to promote viral replication. PLpros of these viruses post-translationally modify host cell proteins and immune mediators such as IFN-β, ISG15 protein, IRF3, TLR3, TLR4, TLR7 to evade innate immunity [13-23]. Drug-like noncovalent inhibitors of PLpros target these proteases for therapeutic purposes [24-29]. These types of inhibitors block PLpro cleavage of viral polypeptides as well as inhibition of its deubiqutinase and deISGylase functions [24-26, 30, 31].

Understanding the features of COVID-19 pathogenesis can aid in drug repurposing and discovery. Angiotensin receptor blockers (ARBs), including losartan, have been thought to be able to provide protection against COVID-19 pathogenesis [32-34]. It is thought that downregulation of ACE2, which occurs during SARS-CoV2 entry, leads to local RAS dysregulation with the increase in angiotensin II/angiotensin (1-7) ratio resulting in pro-inflammatory, pro-apoptotic and pro-thrombotic effects eventuating in COVID-19-induced cytokine storm [34-37]. In this context, human recombinant soluble ACE2 showed promising results in restricting the severe form of COVID-19, yet its short half-life requires further studies to prolong its circulation [38, 39]. Instead of increasing ACE2, selective AT1R antagonism by ARBs has been hypothesized to aid in ameliorating lung pathology in COVID-19 through rebalancing Ang II/angiotensin (1-7) ratio and indirectly promoting Ang II-induced activation of AT2R with antagonistic effect against Ang II activation of AT1R [34, 40]. A recent small randomized clinical trial showed that C21, an agonist of AT2R, could improve mortality rate and respiratory function significantly in hospitalized patients with COVID-19 [41]. However, the use of ARBs was constrained in the early phase of the pandemic due to concerns over their possible role in increasing viral load due to ACE2-upregulating effect of these drugs [42, 43]. Further studies, on the other hand, have shown that ARBs are safe in patients with COVID-19 [44, 45]. This paved the way to conduct experimental and clinical studies on ARBs in COVID-19. An experimental study demonstrated that candesartan, an ARB, could decrease cytokine storm in COVID-19 [46]. In a clinical study on 75 hospitalized patients with COVID-19 who did not need intensive care revealed that candesartan could reduce hospital length of stay, time-to-negative swab test and time-to-improvement of chest X-ray [47]. Another recent multicenter randomized open-label controlled clinical trial on 158 patients showed that 80 mg of telmisartan, another ARB, added to standard of care could improve biochemical and clinical indices with reducing CRP level, ICU admission rate and length of hospital stay compared to a control group [48]. In addition to its effect on RAS, losartan in a recent in silico study was shown to change the structure of ACE2 affecting its binding with the RBD of the SARS-CoV-2 S protein, hence, possibly decreasing the affinity of the virus to its receptor. Moreover, this study showed that losartan induces changes in atomic configuration of SARS-CoV-2 PLpro by occupying the place for inhibitors of PLpro with low docking energy (high affinity) (Fig. 1) [34]. In addition, based on the previous experimental studies, losartan attenuates the inflammatory responses leading to acute respiratory distress syndrome [49].

We sought to validate the bioinformatic findings in our previous in silico study [34] and examined the possible inhibitory effect of losartan against deubiquitinase and deISGylase properties of SARS-CoV-2 PLpro. We also examined if losartan could prevent viral replication of SARS-CoV-2 in pre- and post-infected Vero E6 cells.

MATERIALS AND METHODS

Chemicals and Reagents
Z-RLRGG-7-amino-4-methyl-courmarin (peptide-AMC) was purchased from Bachem. Ubiquitin−7-amino-4-methylcourmarin (Ub-AMC) was purchased from Boston Biochem; human ISG15−7-amino-4-methylcourmarin (ISG15-AMC) was purchased from Boston Biochem. Lys48 linked tetra-Ub were obtained from Boston Biochem; DL-dithiothroitol (DTT) was purchased from GoldBio, and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from GoldBio. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Fisher BioReagents. Imidazole was purchased from Acros Organics; tris(hydroxymethyl)aminomethane (Tris) was purchased from Fisher Scientific. Losartan and Sodium chloride (NaCl) were purchased from Fisher
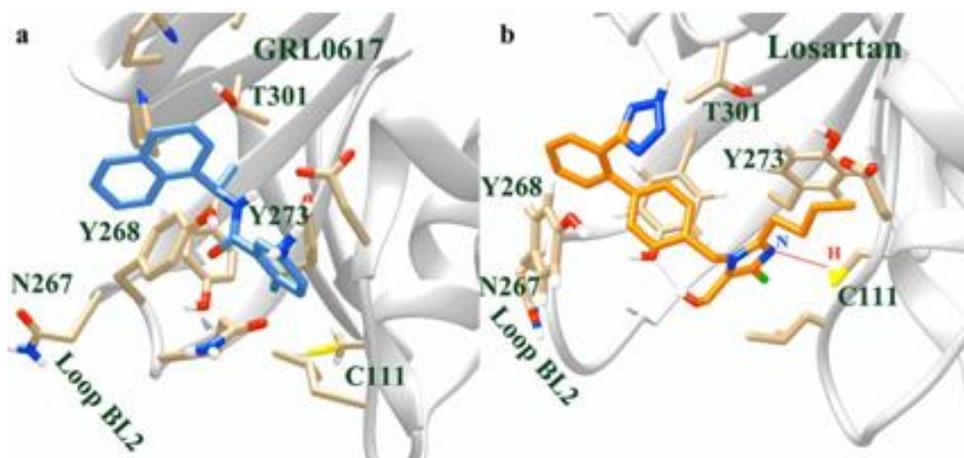
Fig. 1. Positioning of GRL-0617 (PLpro inhibitor) and losartan in the active site of PLpro. (a) X-ray crystallography structure of PLpro and GRL-0617 (PDB ID: 7cmd). (b) Losartan positioning was achieved after 100ns MD simulation. Losartan poses in the same position of the inhibitor (GRL-0617) according to PLpro amino acids in the circumferential area of these two ligands. Note: X-ray crystallography in contrast to NMR analysis and theoretical modeling cannot resolve hydrogen atoms in most protein crystals found in PDB files [50].

Chemical and bovine serum albumin (BSA) was purchased from Sigma Life Science.

Determination of IC$_{50}$ Values

IC$_{50}$ assays were performed using previously described methods for peptide-AMC cleavage experiments [51]. All assays were run using Corning Costar half-volume 96-well plates containing AMC buffer (100 mM NaCl, 50mM HEPES (pH = 7.5). 0.01 mg/mL bovine serum albumin (BSA), and 5 mM DTT) to a final volume of 50μL and performed in triplicate. The CLAIROstar plate reader (BMG Lab Tech, Inc.) was used to measure the fluorescence of the AMC cleavage, and the data was analyzed using MARS (BMG Lab Tech, Inc.). Assays using peptide-AMC substrate contained 1μM SARS-CoV-2 PLpro and 50 μM peptide-AMC in 98% AMC buffer/2% DMSO. Reactions were performed in triplicate with inhibitor concentrations ranging from 390 nM to 2.25 mM. Further assays utilizing ISG15-AMC and Ub-AMC contained 1nM PLpro with 1 μM substrate or 25nM PLpro with 2 μM substrate respectively.

Inhibition of Poly-Ub and proISG15 Cleavage

Lys48 linked tetra-Ub obtained from Boston Biochem was incubated at 10.5 μM with 23 nM SARS-CoV-2 PLpro and 2.25 mM losartan. Reactions were performed in 97.75% AMC buffer/2.25% DMSO at a volume of 80 μL and a temperature of 37°C. 10 μL samples were taken at the indicated time points and heat-shocked at 98°C

for 5 min. SDS-PAGE analysis was performed using Mini-PROTEAN TGX and Coomassie blue. Utilizing similar parameters 10 μM human proISG15 was incubated with 20 nM PLpro and 2 mM losartan. Reactions were performed in 98% AMC buffer/2% DMSO at a volume of 90 μL. 10 μL samples were taken at the indicated time points and heat-shocked at 98°C for 5 min. SDS-PAGE analysis was performed using Mini-PROTEAN TGX Stain-Free.

SARS-CoV-2 and losartan Antiviral Assays.

SARS-CoV-2 (2019-nCoV/USA-WA1/2020; MN985325.1) was received from BEI resources and propagated in Vero E6 cells. SARS-CoV-2 studies were performed in an approved BSL3 facility in the Animal Health Research Center (AHRC) at the University of Georgia. Work followed Biosafety in Microbiological and Biomedical Laboratories (BMBL) guidelines Appendix F5 from the CDC. Infection was done at a multiplicity of infection (MOI) = 0.1 in serum-free in Dulbecco's minimal essential medium (DMEM) for 1h after which the virus-containing media was decanted and replaced with DMEM supplemented with 1% heat-inactivated fetal bovine serum [52]. The virus was propagated for 56 h before it was harvested, and the titer determined by plaque assay on Vero E6 cells [53]. The viral plaques were counted, and the titer was determined as PFU/ml. The Vero cells were plated at 2 x 10^4 cells/well in a 96-well plate and incubated overnight at 37°C. Losartan was prepared in DMEM to 100 μM, 50 μM, 25 μM, 12 μM, 6 μM, 3 μM or 1

μM. The 96-well plate was washed with PBS and the dilutions of losartan added in triplicate. The cells were preincubated for 1 hour, the plate was washed once with PBS and then infected at a MOI = 0.01 for 1 h after which the virus containing media was removed and fresh losartan concentrations were added to the cells in a final volume of 200 μL per well and incubated for 96 h at 37ºC at 5% CO2. Otherwise, cells were plated at 2 x 10^4 cells/well in a 96-well plate, then infected at a MOI = 0.01 for 1 h after which the virus containing media was removed, and media containing 200 μL of diluted losartan added per well and incubated for 96 h in triplicate. These plates were fixed, stained for viral nuclear protein (NP) levels and assayed on a Cellomics ArrayScan. Levels of NP correlates with replication the infected cells [54]. Average percentage responder intensity of the fluorescent channel from NPs of the virus was measured and the calculations were performed using Prism 8 from GraphPad.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.0. IC_{50} and EC_{50} calculations and statistical analysis were performed using Prism 8.0 from GraphPad. P-values < 0.001 are statistically significant. Comparisons between two normally distributed groups were performed by Wilcoxon signed rank test using Prism 8.

RESULTS

Deubiquitinase and deISGylase inhibitory activity of losartan on SARS-CoV-2 PLpro.

To characterize its inhibitory effects on SARS-CoV-2 PLpro, losartan was incubated at various concentrations with SARS-CoV-2 PLpro and a peptide substrate containing the last five consensus amino acids of ubiquitin (Ub) and interferon stimulated gene product 15 (ISG15) in conjugation with a C-terminal 7-amido-4-methylcoumarin (AMC) fluorogenic reporter group (peptide-AMC). These assays revealed IC_{50} value of $1200 \pm 61 \mu M$ for losartan against peptide-AMC (Table 1).

ISG15 is the preferred substrate of CoV2 PLpro [24]. To test its inhibitory effect, losartan was investigated against ISG15-AMC and peptide-AMC. SARS-CoV-2 PLpro has a strong preference for ISG15 over peptide as a substrate displacing competitive inhibitors with lower affinity for ISG15. These data suggest that losartan, due to its structure, may be interacting with elements of PLpro that accommodate both

peptide and Ub-like substrates such as the active site, or the P3/P4 pocket of the PLpro [26]. This pocket is adjacent to the active site and facilitates cleavage of Ubl substrates by binding the C-terminal leucine and arginine of the RLRGG motif [26]. Other small molecule PLpro inhibitors of similar size have been shown to bind in this pocket in both SARS-CoV-1 and SARS-CoV-2 [25].

Efficacy of losartan for Inhibiting CoV2 PLpro.

To explore the relationship between substrate affinity and inhibitor efficacy, losartan was tested against Ub-AMC at 200μM. SARS-CoV2 PLpro has a strong preference for ISG15 over Ub as a substrate [24], where the catalytic efficiency of the PLpro for Ub is approximately one-tenth that of ISG15 but more than 250 times that of the peptide-AMC. Therefore, competitive non-covalent inhibitors limit Ub-AMC cleavage at a rate somewhere between those of ISG15-AMC and peptide-AMC. Losartan had an inhibition rate of 2.3% when tested against Ub-AMC (Table 1). Inhibition rate of losartan was 6.9% against ISG15 cleavage (Table 1). These differences between inhibitory rates of losartan against ISG15-AMC and Ub-AMC are unlikely to have a qualitative difference in a biological system. To confirm this, molecular weight shift assays were performed with more biologically accurate substrates.

Effect of losartan on Tetra-Ub Deubiqutination.

Ubl substrates with AMC tags at their C-terminal glycine are not entirely reflective of natural substrates. Also, PLpros have been observed to prefer cleaving poly-Ub chains over mono-Ub substrates [55]. To determine if the inhibitory values derived from AMC cleavage assays are reflective of a qualitative change in DUB and deISGylase activity, losartan was tested against human proISG15 and K48 linked tetra-Ub. As expected, losartan at 2 mM shows a small reduction deISGylase activity relative to the control (Fig. 2).However, losartan at 2.25 mM effectively eliminated DUB activity (Fig. 3).

Activity of losartan on SARS-CoV-2 replication.

Losartan treatment of Vero E6 cells showed a dose-dependent (0 - 100 μM concentrations) and significant (p<0.001) reduction in SARS-CoV-2 replication in both pre- and post-infection studies (Fig. 4). The half- maximal effective concentration

Table 1. Percentile of inhibition and IC_{50} of cleavage of peptide-AMC, percentile of inhibition of ISG15-AMC and Ub-AMC in the presence of 200µM losartan. IC_{50} of losartan for cleavage of peptide-AMC is 1200±61 µM. IC_{50} of ISG15 and Ub were not determined needing high concentrations of losartan

	Peptide (200µM)	PeptideIC_{50} (µM)	ISG15 (200µM)	Ub (200µM)
Losartan	35.1% ± 2.7	1200 ± 61	6.9% ± 3.4	2.3% ± 1.4

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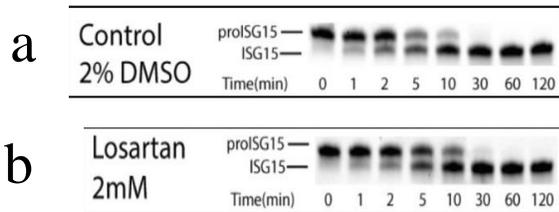
Fig. 2. Gel shift analysis. Inhibitory activity of losartan on deISGylation of PLpro. (a) deISGylation of PLpro on proISG15 in 10 µM of proISG run against 20 nM of SARS-CoV-2 PLpro, at 37°C, over 2 hours with samples taken at the indicated intervals with Gel cleavage assay visualized by Commassie Blue Staining. Strong PLpro deISGylation is noted. (b) Losartan, at lower concentrations up to 2mM concentration added to proISG15 and PLpro solution, demonstrates insignificant inhibitory effect on PLpro deISGylation between 5 and 30 minutes.
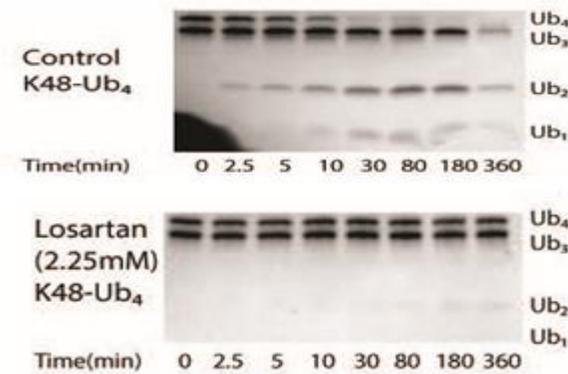
Fig. 3. Inhibitory effect of losartan on deubiquitination activity of PLpro on K48-Ub_4 (a) At the mentioned intervals over 6 hours, multiple ten µL samples of Lys48 linked tetra Ub at 13.65 µM with 23 nM CoV2 PLpro in AMC buffer at 37 °C were taken and heat-shocked at 98 °C for 5 minutes. Gel cleavage assay visualized by Commassie Blue Staining shows PLpro's cleavage of Ub_4 to mostly DUB and some monoUb. (b) Addition of losartan, at 2.25 mM concentration, demonstrates a significant inhibitory effect on PLpro deUbiquitination of Ub_4. Due to the low inhibitory effect of losartan on Ub

(2.3% at 200 µM in Table 1) 2.25 mM was used in this experiment.
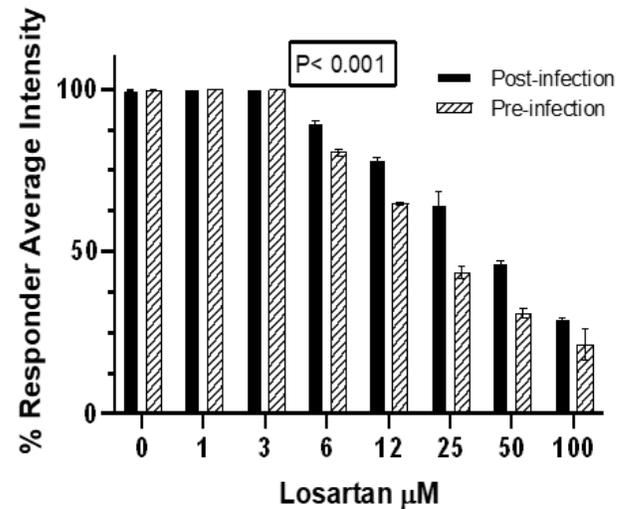
Fig. 4. Losartan treatment, in a dose-dependent manner (1-100 µM), decreases CoV2-infected Vero E6 cells pre-infection more significantly than post-infection (Wilcoxon test p<0.001).
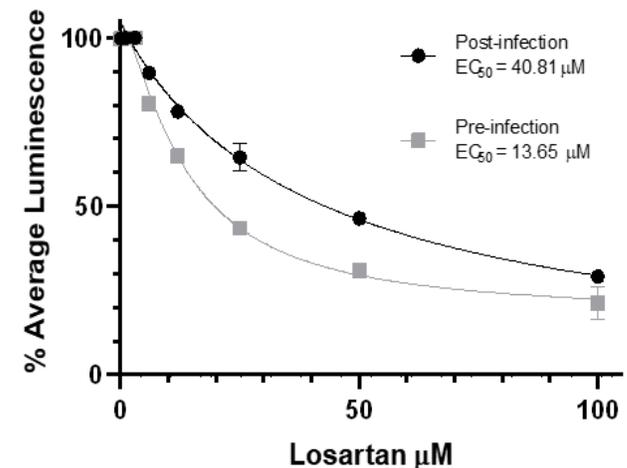
Fig. 5. SARS-CoV-2 replication in pre- and post-infection treatment. Losartan dose-dependently reduces the production of viral nuclear proteins in Vero E6 cells (80%). EC_{50} curves are significantly different in pre- and post-infection groups (P<0.001).

(EC_{50}) of losartan significantly (p<0.001) decreased SARS-CoV-2 replication using 41 µM in post-infection treatment and 13.7 µM in the pre-infection treatment (Fig. 5).

DISCUSSION

Replication of SARS-CoV-2 depends on PLpro and Mpro functions [31]. Inhibitors of SARS-CoV-1 PLpro, in several studies, have been shown to be as effective against SARS-CoV-2 PLpro [56, 57]. Although many of the findings were promising, the development in introducing approved drugs is lagging. Our *in vitro* study revealed that losartan was a weak inhibitor of PLpro in cleavage of peptide-AMC ($IC_{50}=1200\pm61\mu M$). It did not substantially suppress deISGylating ability of PLpro (Fig. 3). It is possible that losartan interacts with the P3/P4 pocket of PLpro adjacent to its active site and is displaced in the presence of ISG15. This pocket could facilitate cleavage of Ubl substrates by binding the C-terminal of leucine and arginine, of the RLRGG motif, through a protonating reaction in a cleft in the PLpro containing a conserved triad of Histidine(H272)-Cysteine(C111)-Aspartic Acid(D286) in papain-like proteases [26, 58, 59].

It has been reported that SARS-CoV-2 PLpro prefers deISGylation ten times more than deubiquitination [24]. However, our study demonstrated that losartan in 2.25mM concentration could completely suppress cleavage of K48 linked polyubiquitin chains by PLpro (Fig. 4). This underscores that due to PLpro substrate affinity, a PLpro's viral evasion mechanism facilitated by their deubiquitinase activities may be impacted at lower competitive inhibitor concentrations than that of their deISGylase activities.

Considering the putative effects of losartan in the treatment of COVID-19, we assessed its impact on SARS-CoV-2 replication in Vero E6 cells. Vero E6 cells are derived from the kidney of African green monkeys and express ACE2 [60-62]. These cells do not produce IFN type I allowing SARS-CoV-1 and SARS-CoV-2 replication without IFN inhibition [63-67]. In our study, losartan treatment prevented SARS-CoV-2 replication. At the highest losartan concentrations, pre-infection and post-infection treatment prevented SARS-CoV-2 replication by 80% and 70%, respectively (Figures 5 and 6). Based on EC_{50}, when used prior to infecting the cells (pre-infection), losartan with an EC50 of 13.6µM reduced SARS-CoV-2 replication by more than 50% compared to post-infection treatment with this drug

with an EC_{50} of 40.8 µM. The effect of losartan in pre-infection is in agreement with a previously published analysis showing SARS-CoV-2 had a lower affinity for ACE2 in the presence of losartan [34].

In addition to our experimental study ongoing clinical trials are assessing the efficacy of losartan in the treatment of COVID-19. Data from future robust clinical trials combined with the data from this study will validate whether globally available and inexpensive ARBs could improve the management of COVID-19.

CONCLUSION

Losartan showed weak inhibitory effect on viral PLpro deubiquitinase and deISGylase properties. Importantly, losartan treatment of Vero E6 cells prior to and after SARS-CoV-2 infection inhibited the virus replication. Additional experiments are needed to better assess the structural changes in the viral proteins and their biological products when exposed to losartan. Outcome of these experiments may provide a viable pathway for antiviral design and development. Ongoing and future randomized clinical trials in different countries with well diversified cohort would significantly aid in our understanding the effect of losartan on the course of COVID-19. Losartan with its *in vitro* inhibitory effect on viral replication and its significant benefits in cell protection without cell toxicity may play an important role in slowing the spread of the disease, medical management of individuals unresponsive to vaccination or new mutations, easing healthcare cost and burden in communities globally.

CONFLICTS AND FINANCIAL INTERESTS
No conflict of interest to report.

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