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Research paper

# Discovery of chebulagic acid and punicalagin as novel allosteric inhibitors of SARS-CoV-2 3CL$^{pro}$

Ruikun Du [a,b,c,1], Laura Cooper [d,1], Zinuo Chen [a], Hyun Lee [e], Lijun Rong [d,**], Qinghua Cui [a,b,c,*]

[a] College of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan, 250355, China
[b] Experimental Center, Shandong University of Traditional Chinese Medicine, Jinan, 250355, China
[c] Qingdao Academy of Chinese Medicinal Sciences, Shandong University of Traditional Chinese Medicine, Qingdao, 266122, China
[d] Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL, 60612, USA
[e] Department of Pharmaceutical Sciences, Center for Biomolecular Sciences, College of Pharmacy, Biophysics Core at Research Resources Center, University of Illinois at Chicago, Chicago, IL, 60607, USA

## ARTICLE INFO

## ABSTRACT

The emerging SARS-CoV-2 infection is the cause of the global COVID-19 pandemic. To date, there are limited therapeutic options available to fight this disease. Here we examined the inhibitory abilities of two broad-spectrum antiviral natural products chebulagic acid (CHLA) and punicalagin (PUG) against SARS-CoV-2 viral replication. Both CHLA and PUG reduced virus-induced plaque formation in Vero-E6 monolayer at noncytotoxic concentrations, by targeting the enzymatic activity of viral 3-chymotrypsin-like cysteine protease (3CL$^{pro}$) as allosteric regulators. Our study demonstrates the potential use of CHLA and PUG as novel COVID-19 therapies.

## 1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the virus that causes a pneumonia like illness pandemic called coronavirus disease 2019 (COVID-19). The disease has reached to almost every country in the world, posing a serious threat to global public health and the economy (Hamid et al., 2020). As of March 4, 2021, more than 114.43 million cases have been confirmed with 2.54 million deaths globally (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Encouragingly, 12 COVID-19 vaccines have been approved and globally available, and 22 vaccine candidates are in phase III clinical trials (https://covid19.trackvaccines.org/vaccines/). However, evidence is increasing that SARS-CoV-2 variants could evade immune responses triggered by either vaccines or previous infections, which may compromise the vaccine effectiveness (Callaway, 2021; Tegally et al., 2020). On the other hand, there is no specific antiviral therapy available for clinical usage, except remdesivir, of which the therapeutic effect is still under debate (Beigel et al., 2020). There is a pressing need to discover additional effective antivirals for the treatment of SARS-CoV-2 infection for use alone or in combination with already approved antiviral therapies.

Broad spectrum antiviral agents are valuable candidates of inhibitors against emerging viruses (Zhang et al., 2020b). To our knowledge, two hydrolyzable polyphenolics, chebulagic acid (CHLA, Fig. 1A) and punicalagin (PUG, Fig. 1B), have been well acknowledged as cost-effective and broad-spectrum antivirals. Previous studies have demonstrated CHLA and PUG can block interactions between cell surface glycosaminoglycan (GAG) and viral glycoproteins. Through this mechanism, they inhibit infections by diverse viruses that employ GAGs for host cell entry, such as herpes simplex virus type 1, human cytomegalovirus, and hepatitis C virus (Lin et al., 2011, 2013). Moreover, we recently identified that CHLA and PUG possess inhibitory effects against influenza viruses by targeting neuraminidase mediated virus release (Li et al., 2020b, 2020c).

In this study, we investigated the potent antiviral activity of CHLA and PUG against SARS-CoV-2.

## 2. Materials and methods

### 2.1. Virus and reagents

The clinical isolate of SARS-CoV-2 (SARS-CoV-2, Isolate USA-WA1/

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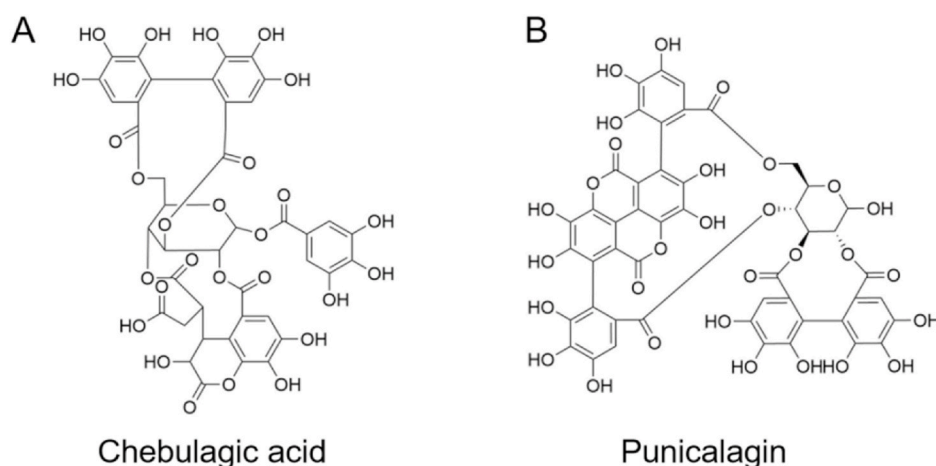
**Fig. 2. CHLA and PUG inhibit SARS-CoV-2 replication**. A. Plaque reduction assay of CHLA and PUG as well as remdesivir (3 μM) against authentic SARS-CoV-2 at indicated concentrations. B. Dose-dependent inhibition of CHLA and PUG on SARS-CoV-2 replication. Antiviral activity and cytotoxicity are shown in red and blue, respectively. The $EC_{50}$ and $CC_{50}$ are displayed in the upper left corner for each compound. The data represent mean ± standard deviation (SD) of the triplicate measurements. $IC_{50}$ values were determined by fitting the dose-response curves with four-parameter logistic regression in Prism GraphPad (version 8.1.2). All data was normalized to virus alone.
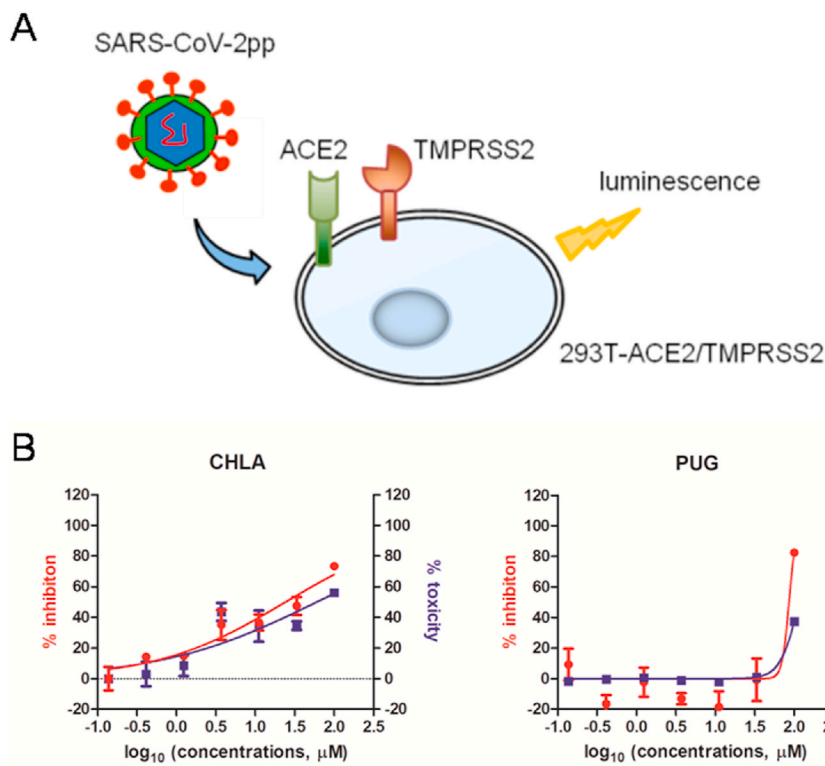


**Fig. 3. Pseudoviral infection assay**. A. representative diagram of pseudo-SARS-CoV-2 assay, where 293T cells transiently expressing ACE2 and TMPRSS2 were used as target cells. B. anti-pseudoviral activity and cytotoxicity are shown in red and blue, respectively. The data represent mean ± standard deviation (SD) of the triplicate measurements.

glycoproteins, we therefore first tested whether CHLA and PUG act by blocking SARS-CoV-2 entry using a pseudotyped SARS-CoV-2 (SAR-S-CoV-2pp). However, upon adding CHLA or PUG during infection of SARS-CoV-2pp into target 293T cells transiently expressing ACE2 and TMPRSS2 (Fig. 3A), neither CHLA nor PUG inhibited pseudovirus entry without interfering with target cell viability, indicating that CHLA and PUG inhibit SARS-CoV-2 replication by a mechanism other than preventing S-mediated viral entry (Fig. 3B).

### 3.3. CHLA and PUG exhibited inhibitory effects against 3CLpro activity

The 3-chymotrypsin-like cysteine protease ($3CL^{pro}$) enzyme is one of the best characterized drug targets among coronaviruses (Zhang et al., 2020a). Along with the papain-like protease ($PL^{pro}$), $3CL^{pro}$ is an essential viral protease that processes the viral polyproteins, therefore, inhibiting the activity of this enzyme would block viral replication (Dai et al., 2020). Interestingly, an *in silico* study revealed that several hydrolyzable polyphenolics including CHLA are potential inhibitors of the SARS-CoV-2 $3CL^{pro}$ (Khalifa et al., 2020). To address this, CHLA and PUG were subjected to a SARS-CoV-2 $3CL^{pro}$ enzymatic inhibition assay. Upon mixture of $3CL^{pro}$ with a FRET-based peptidic substrate in reaction buffer, the substrate gets hydrolyzed. After hydrolysis, the fluorophore Edans is no longer affected by the quencher molecule Dabcyl, resulting in an increase of fluorescence signal with proper filters (Fig. S1A). Both CHLA and PUG dose-dependently reduced fluorescence production (Fig. S1B), with $IC_{50}$ values of $9.09 \pm 0.87$ μM and $4.62 \pm 0.27$ μM, respectively (Fig. 4). A fluorescent interference assay further illustrated that CHLA and PUG affected fluorescence only at higher concentrations (>50 μM, data not shown). Taken together, our study revealed that CHLA and PUG can block the enzymatic activity of $3CL^{pro}$. Of note, CHLA showed little effect on the enzymatic activity of SARS-CoV-2 $PL^{pro}$, while PUG slightly inhibits the $PL^{pro}$ activity with $IC_{50}$ of over 50 μM (Fig. S2). These data suggest that PUG is a potential dual-target SARS-CoV-2 inhibitor, although its inhibitory effect against the $PL^{pro}$ is weak.

### 3.4. CHLA and PUG exhibited reversible, noncompetitive inhibition against 3CLpro

To better understand the kinetic modes involved in the interaction of CHLA and PUG with SARS-CoV-2 $3CL^{pro}$, the proteolytic activity of the enzyme was measured using a series of enzyme concentrations and at various inhibitor concentrations. The substrate concentrations were held constant at sub-saturation levels. At each concentration of test inhibitor, the initial reaction velocities were plotted with the concentrations of $3CL^{pro}$, and fitted to a line using linear regression analysis. As

indicated by Fig. 5A, as the concentration of either CHLA or PUG increases, the slope of the line decreases, suggesting that both CHLA and PUG are reversible inhibitors. In order to deduce the mode of binding, Lineweaver-Burk plots were carried out at a constant concentration of $3CL^{pro}$ (250 nM), increasing concentrations of substrate, and in absence or presence of various concentrations of CHLA or PUG. As a result, both CHLA and PUG exhibited noncompetitive modes of inhibition since all lines intercepting the x-axis (Fig. 5B).

### 3.5. Molecular docking

According to above enzymatic assays, both CHLA and PUG are noncompetitive allosteric inhibitors. In order to search potential allosteric binding sites, *in silico* docking of CHLA and PUG to $3CL^{pro}$ was thereafter performed using AutoDock Vina software. As depicted in Fig. 6, both CHLA and PUG may interact with the cleft between domain II and domain III within $3CL^{pro}$ with stable binding free energy. This binding site is located a little away from the substrate binding pocket, where His41 and Cys145 form the catalytic dyad (Dai et al., 2020).

## 4. Discussion

In the urgent campaign to develop SARS-CoV-2 therapeutics, natural products from medicinal plants have been an important source of new lead compounds exhibiting different mechanisms of action, such as shikonin (Li et al., 2020a), salvianolic acid C (Yang et al., 2020), cepharanthine (Rogosnitzky et al., 2020), lycorine (Zhang et al., 2020b), ginkgolic acid (Chen et al., 2021) and so on (Zhang et al., 2020c). We herein report two additional natural products, CHLA and PUG as novel SARS-CoV-2 inhibitors. By binding to SARS-CoV-2 $3CL^{pro}$ at a pocket other that substrate binding site, CHLA and PUG act as allosteric inhibitors in reversible, noncompetitive manner.

There are two classes of inhibitors against a cysteine protease, covalent inhibitors and non-covalent ones. Development of covalent inhibitors is usually challenging due to their toxicity and lack of specificity resulting from covalent modification of untargeted cysteine residues, while encouraging strategies have been explored, for example, by rational design, to inhibit $3CL^{pro}$ via its covalent inhibition (Dai et al., 2020; Jin et al., 2020; Qiao et al., 2021).

On the other hand, a non-covalent inhibitor may specifically bind to the target proteins reversibly. Compared to covalent inhibitors, non-covalent inhibitors can often be optimized to diffuse through membranes and bind to target proteins with high affinity (Zhang et al., 2015). Moreover, non-covalent inhibitors are often more chemical stable than their covalent counterparts since they don't have reactive warheads,and undesirable toxic effects can be reduced for their binding to host
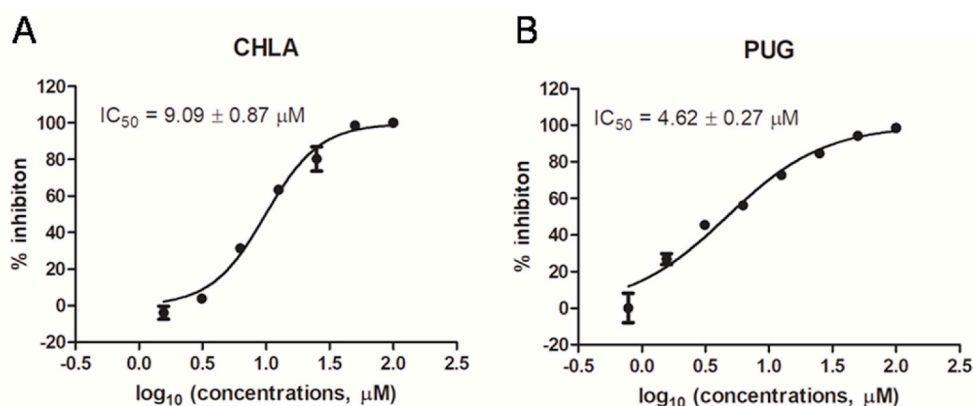


**Fig. 4. Fluorescence resonance energy transfer (FRET)-based $3CL^{pro}$ enzymatic inhibition assay**. The inhibitions of CHLA (A) and PUG (B) against $3CL^{pro}$ were determined using a FRET-based cleavage assay. For each compound, the IC50 value is displayed in the upper left corner. The data represent mean ± standard deviation (SD) of the triplicate measurements.
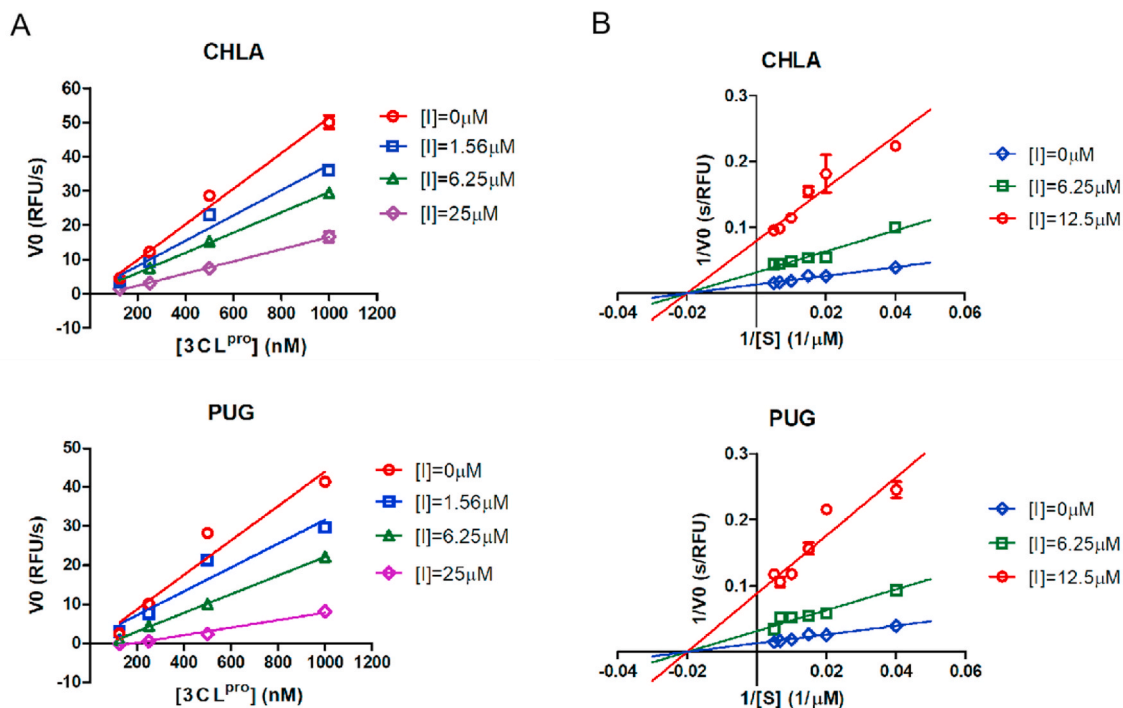
**Fig. 5. Graphical determination of the type of enzymatic inhibition.** A. Relationship of the enzymatic activity of SARS-CoV-2 3CL$^{pro}$ as function of enzyme concentrations at different concentration of CHLA or PUG. B. Lineweaver-Burk plot for inhibition of CHLA or PUG on SARS-CoV-2 3CLpro.
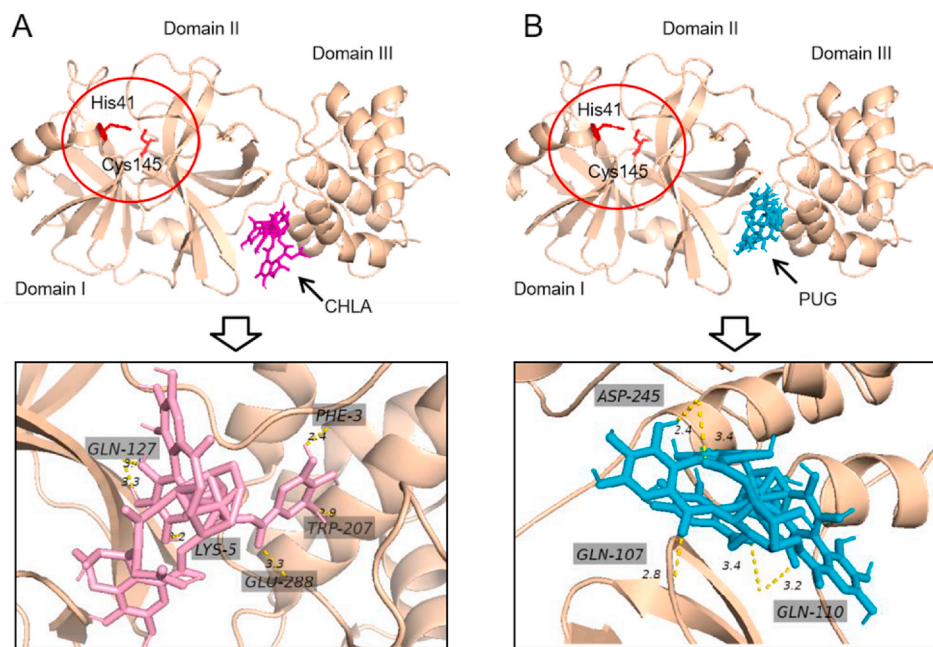


**Fig. 6. Predicted binding modes of CHLA (A) and PUG (B) to 3CL$^{pro}$ (PDB ID: 6m2n).** The red circle indicates the catalytic site. The SARS-CoV-2 3CL$^{pro}$ is present in wheat cartoon, while CHLA and PUG are shown as sticks. The bonds are represented as the dashed lines, with the bond length measured between heavy atoms.

proteins and nucleic acids are reversible (El-Baba et al., 2020).

Virtual screens have placed hundreds chemically diverse molecules in the active site of 3CL$^{pro}$, which is the main target for a non-covalent inhibitor (Douangamath et al., 2020; Gyebi et al., 2020; Khalifa et al., 2020). Although most of the predicted inhibitors either were not subjected to antiviral assays or failed to inhibit SARS-CoV-2 replication, compound ML188 has been validated as SARS-CoV-2 inhibitor by competitively binding to 3CL$^{pro}$ with natural substrate (Lockbaum et al., 2021). Besides, a non-covalent inhibitor may also act by binding to

allosteric sites, which are away from the active site (El-Baba et al., 2020). Recently, a highly reactive pocket in the dimerization region at the domain III apex of SARS-CoV-2 3CL$^{pro}$ has been recognized as a possible allosteric site. Interestingly, our *in silico* docking data clearly reveal that both CHLA and PUG accommodates this pocket (Fig. 6).

Historically, in order to discover non-covalent cysteine protease inhibitors, reducing reagents were usually added in the enzymatic assay of cysteine protease to prevent nonspecific covalent modification of the catalytic cysteine (Ma et al., 2020a; Ratia et al., 2008). However, in the

case of CHLA and PUG, addition of 4 mM DTT in the enzymatic assay of SARS-CoV-2 3CL$^{pro}$ shifted the IC$_{50}$ curves to right by 7–10 folds, suggesting significant loss of inhibitory effects (Fig. S3). This is inconsistent with our finding that CHLA and PUG are reversible, noncompetitive inhibitors of 3CL$^{pro}$. To address this discrepancy, we suspect that reducing reagents might induce slight conformational changes of 3CL$^{pro}$, which would not affect the detectable enzymatic activity, while the cavities where an allosteric inhibitor binds might be impaired. Although more evidence is required to support this hypothesis, the scientific community should be cautious to add reducing reagents in the enzymatic assay of cysteine protease if allosteric inhibitors were anticipated.

In summary, our study demonstrates CHLA and PUG as a novel class of SARS-CoV-2 inhibitors, by allosterically regulating the 3CL$^{pro}$ activity.

## Declaration of competing interest

The authors declare no conflict of interest.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2021.105075.

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