



Research Article

Oridonin inhibits SARS-CoV-2 replication by targeting viral proteinase and polymerase



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ABSTRACT

COVID-19 has become a global public health crisis since its outbreak in China in December 2019. Currently there are few clinically effective drugs to combat SARS-CoV-2 infection. The main protein (M^{pro}), papain-like protease (PL^{pro}) and RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2 are involved in the viral replication, and might be prospective targets for anti-coronavirus drug development. Here, we investigated the antiviral activity of oridonin, a natural small-molecule compound, against SARS-CoV-2 infection *in vitro*. The time-of-addition analysis showed that oridonin efficiently inhibited SARS-CoV-2 infection by interfering with the genome replication at the post-entry stage. Mechanistically, the inhibition of viral replication by oridonin depends on the oxidation activity of α , β -unsaturated carbonyl. Further experiments showed that oridonin not only effectively inhibited SARS-CoV-2 M^{pro} activity, but also had some inhibitory effects on PL^{pro}-mediated deubiquitinating and viral polymerase-catalyzed RNA elongation activities at high concentrations. In particular, oridonin could inhibit the bat SARS-like CoV and the newly emerged SARS-CoV-2 omicron variants (BA.1 and BA.2), which highlights its potential as a pan-coronavirus antiviral agent. Overall, our data provide strong evidence that oridonin is an efficient antiviral agent against SARS-CoV-2 infection.

1. Introduction

The novel coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global public health crisis since its outbreak in December 2019. As of March 2023, more than 220 countries and territories around the world have reported at least 680 million confirmed cases of COVID-19, including more than 6.8 million deaths (<https://www.worldometer.info/coronavirus/>). SARS-CoV-2 is an enveloped, single-stranded, positive-sense RNA virus with a genome size of ~30 kb, belonging to the genus *Betacoronavirus* from the *Coronaviridae* family (Zhou et al., 2020). The viral RNA contains at least six open reading frames (ORFs) encoding two overlapping polyproteins precursors (pp1a and pp1ab), four structural proteins (spike, envelope, membrane, and nucleocapsid),

and nine accessory proteins (Wu et al., 2020; Zhou et al., 2020). Two large polyproteins (pp1a/pp1ab) are cleaved into 16 nonstructural proteins (nsps) essential for transcription and viral genome replication by two viral cysteine protease, the papain-like protease (PL^{pro}) and the main protease (M^{pro}) (Freitas et al., 2020; Jin et al., 2020).

SARS-CoV-2 M^{pro} is a homodimer protease. It consists of three domains and has an active site which is located within the cleft formed between domain I and domain II. The active site of M^{pro} has a histidine-cysteine catalytic dyad that consists of the conserved residues H41 and C145 (Dai et al., 2020). It recognizes the Leu-Gln ↓ (Ser/Ala/Gly) sequence in polyproteins, which is a characteristic motif and shared among the coronaviruses, generating nsp4–16 proteins. Since it is highly conserved across the *Coronaviridae* family and has no closely related human homologues, the M^{pro} can be considered as a prospective target

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for anti-coronavirus drug development (Zhang Y. et al., 2022). Several co-crystal structures of M^{pro} with the inhibitors have been reported, providing potential opportunities for structure-based drug design and virtual screening (Dai et al., 2020; Fu et al., 2020; Jin et al., 2020; Ma et al., 2020b; Sacco et al., 2020; Vuong et al., 2020; Ampornpanai et al., 2021). Recently, several SARS-CoV-2 M^{pro} inhibitors, such as Paxlovid, have been approved for clinical use (Owen et al., 2021) and other promising candidates, S-217622 and FB2001, are currently in clinical development (Shang et al., 2022; Unoh et al., 2022). However, the development of novel drug candidates is still needed in reducing drug toxicity, increasing tolerability and broad-spectrum antiviral activity.

Natural compounds, especially medicinal herbs and their extracts, have attracted great attention due to their potential biological activity. In the past 30 years, more than 50% of the newly approved drugs were natural compounds or synthetic compounds based on or inspired by natural compounds (Newman and Cragg, 2016; Chopra and Dhingra, 2021). As a rich resource for anti-SARS-CoV-2 drugs discovery and development, many natural compounds have been evaluated for their activities against SARS-CoV-2 infection (Zhang et al., 2020; Zhang Z. et al., 2022). It has shown that some natural compounds, such as terpenoids and flavonoids, are capable of inhibiting the activity of SARS-CoV-2 M^{pro}, thereby exhibiting good antiviral activities (Gyebi et al., 2021; Kumar et al., 2021; Mandal et al., 2021; Sun et al., 2021).

In present study, we aimed to systematically evaluate the antiviral efficacy of oridonin against SARS-CoV-2 and clarify the antiviral targets in order to provide a reliable theoretical basis for the clinical application of oridonin.

2. Materials and methods

2.1. Cell lines, viruses, antibodies and reagents

Vero-E6 cells (ATCC CRL-1686), BHK-21 (ATCC CCL-10) and Caco-2 cells (ATCC HTB-37) were incubated at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen) containing 10% (vol/vol) fetal bovine serum (FBS, Gibco Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Beyotime, Shanghai, China). SARS-CoV-2 (WIV04 strain, GenBank No: MN996528.1), Bat SARS-like CoV (WIV1 strain, GenBank No: [KF367457.1](#)) and SARS-CoV-2 Omicron variant (BA.1 strain, CSTR: 16533.06.IVCAS 6.7600 and BA.2 strain, CSTR: 16533.06.IVCAS 6.7617) were provided by the National Virus Resource, Wuhan Institute of Virology, Chinese Academy of Sciences. These viruses were propagated in Vero-E6 cells and stored aliquots at –80 °C for experiments. The viral titer was determined by plaque assay. The rabbit anti-RP3-CoV NP protein antibody which is cross-reactive with the NP protein of SARS-CoV-2 was kindly provided by Prof. Zheng-Li Shi (Wuhan Institute of Virology, CAS). Oridonin were purchased from Weikeqi Biotech (Sichuan, China). SARS-CoV-2 nsp7, nsp8 and nsp12 proteins were home-made as described previously (Wang Q. et al., 2020). SARS-CoV-2 PL^{pro} and K48-Ub3 protein were provided by Prof. Jie Zheng from Shanghai Institute of Materia Medica.

2.2. Plaque assay

Virus titration of SARS-CoV-2 was performed by monolayer plaque assay as described previously (Zhang et al., 2020). Briefly, Vero-E6 cells in 24-well plate were incubated with aliquots from serial 10-fold dilutions for 1 h at 37 °C. After adsorption, virus supernatants were removed and monolayer were overlaid with a DMEM mixture consisting of 1% methylcellulose, 2% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were incubated at 37 °C with 5% CO₂ for 4 days for plaque development. Then the overlay was removed and cells in plates were fixed with 3.7% formaldehyde for 24 h and stained with 1% crystal violet in water. Plaques were counted after washing under running water.

2.3. Indirect immunofluorescence (IFA) assay

The cells were seeded on a Chamber Slide (Nalge Nunc). At time points of sample collection, the cells were fixed with cold (–20 °C) 5% acetone in methanol at 25 °C for 10 min, washed three times with PBS and fixed with 3.7% formaldehyde for 24 h. For the detection of viral replication, the cells were incubated with rabbit antibody against RP3-CoV NP protein (1:1000 dilution with PBS) for 1 h. After washing with PBS three times, the cells were incubated with FITC-conjugated goat anti-mouse IgG (1:125 dilution with PBS, Protein Tech Group) at room temperature for 1 h. Following PBS washing, the slides were mounted with 95% glycerol and analyzed under a Zeiss fluorescence microscope.

2.4. Western blot analysis

At 12 h post infection (h.p.i.), the cells from the time of addition assay were lysed in RIPA buffer (Beyotime) on ice for 10 min, followed by denaturation in 5× SDS loading buffer at 95 °C for another 15 min. The samples were analyzed by SDS-PAGE electrophoresis and transferred to PVDF membrane. After being treated with skimmed milk for 2 h, PVDF membrane was successively incubated with primary antibodies against SARS-CoV-2 NP or β-actin and secondary horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit IgG at 25 °C for 1 h. Finally, the PVDF membrane was incubated with a mixed substrate (Pierce ECL Western Blotting Substrate kit), and the corresponding signal is detected and analyzed by the chemiluminescence system (ChemiDoc; Bio-Rad).

2.5. Quantitative real-time PCR (qRT-PCR)

The supernatant of virus-infected cells was heat-inactivated at 65 °C for 30 min, and then the viral RNA was obtained by executing the manufacturer's protocol provided by QIAamp viral RNA mini kit (52906, Qiagen). qRT-PCR were performed using Luna® Universal Probe One-Step RT-PCR Kit (E3006). For genome RNA quantification, the primer pair and probe were used: RBD-qF1: 5'-CAATGGTTTAAACAGGCACAGG-3'; RBD-qR1: 5'-CTCAAGTGTCTGTGGATCAGC-3'; Probe: ACAGCATCAGTAGTGTCAGCAATGTCTC.

2.6. Luciferase assay

The infected cells in 12-well plates were washed once with PBS and lysed with 200 µL lysis buffer (Promega) and subjected to the luciferase assay according to the manufacturer's protocol. Briefly, 20 µL cell lysate was incubated with 50 µL substrate. Mix slightly and measure its relative light unit (RLU) by a multimode microplate reader (Varioskan Flash; Thermo Fisher). All luciferase assays were repeated three times independently.

2.7. Cytotoxicity assays

Vero-E6 cells were seeded in 96-well plates (1 × 10⁴ cells per well) for cytotoxicity assays. After one day cultivation, compound diluents (2-fold) in different concentrations were added to the cells respectively, repeated three times independently. At 24 h post treatment, the cells were incubated with 10 µL CCK8 reagent (cell counting kit-8, Bimake) for 1 h at 37 °C. The absorbance at 450 nm was measured by a multimode Microplate Reader (Varioskan Flash, Thermo Fisher). Cell activity was expressed as the percentage of the absorption value of the treated cells to the untreated cells. For each concentration, mean values of the cell viability were calculated. The CC₅₀ was calculated by nonlinear regression using GraphPad Prism 8.0 software to determine the cytotoxic concentration at which 50% of the cells are viable.

2.8. Antiviral assay using SARS-CoV-2

Vero-E6 or Caco-2 cells were seeded in 24-well plates (8×10^4 cells per well) for antiviral assay. After one day cultivation, the cells were incubated with compound diluents (2-fold) in different concentrations and infected with SARS-CoV-2 (MOI = 0.01) at 37 °C for 24 h or 36 h. Then, the infected-cells supernatants were collected and used for viral RNA extraction to quantify the viral copies. The antiviral activity of compounds was expressed as 50% effective concentration (EC₅₀) and calculated by GraphPad Prism software 8.0.

2.9. Time-of-addition assay

To preliminarily determine which part of the viral life cycle the compound inhibits, time-of-addition assay was conducted. Vero-E6 cells were seeded in 24-well plates (8×10^4 cells per well). After one day cultivation, the cells were treated with oridonin (10 μmol/L), chloroquine (10 μmol/L) or DMSO during the following period: full-time-infection (–1–12 h), during-time-infection (0–2 h), and post-time-infection (2–12 h). The DMSO-treatment group of the final selected studies was consistent with that of the “full-time” group. For all the experimental groups, the cells were infected with SARS-CoV-2 (MOI = 0.05) at 37 °C for 12 h. Viral RNA in cell supernatant was quantified by qRT-PCR assay. Inhibition rates are calculated as “1 – the percentage of viral RNA copies in treatment group relatively to that of control”.

2.10. Transient replicon assay

The inhibitory effect of oridonin on SARS-CoV-2 RNA replication was determined by SARS-CoV-2 replicon carrying a *Renilla luciferase* gene (Zhang Q. et al., 2021). The replicon RNA was electroporated into BHK-21 cells by a GenePulser Xcell system (Bio-Rad, Hercules, CA) as previously described (Zhang Q. et al., 2021). Briefly, 10 μg replicon RNA was added to a 4-mm cuvette containing 0.8 mL of BHK-21 cells (8×10^6). Three electrical pulses were given in 850 V and 25 μF. After 10 min recovery, the transfected cells were seeded in a 12-well plate (3×10^5 cells per well), and immediately treated with oridonin (10 μmol/L), remdesivir (10 μmol/L) or DMSO. At various time points post transfection, the cells were used for luciferase assay.

2.11. *In vitro* M^{pro} inhibition assay

To further verify whether the compound limited viral replication by inhibiting M^{pro} activity, *in vitro* experiments were carried out using the M^{pro} assay kit as the manufacturer's protocol (Beyotime). Briefly, 93 μL Assay Reagent containing SARS-CoV-2 M^{pro} with or without DTT (4 mmol/L) was incubated with 5 μL positive compound GC376, DMSO and the compound diluents in different concentrations at 37 °C for 10 min. Then 2 μL substrate was added to the mixture and incubated in dark at 37 °C for 10 min and the fluorescence was measured using a multimode Microplate Reader. The excitation wavelength is 329 nm and the emission wavelength is 393 nm. The experimental group using Assay Reagent without protease was used as blank control. Calculate the average fluorescence value of each sample well and blank control well, which can be recorded as RFU_{blank control}, RFU_{100% enzyme activity control}, RFU_{positive control} and RFU_{sample} respectively. Inhibition rates were calculated as the percentage of “(RFU_{100% enzyme activity control} – RFU_{sample})/(RFU_{100% enzyme activity control} – RFU_{blank control})”.

2.12. *In vitro* primer-dependent RdRP assay for SARS-CoV-2

To characterize the intervention effects of oridonin, a typical 20-μL reaction mixture containing 6 μmol/L nsp12, 6 μmol/L nsp7, 12 μmol/L nsp8, different concentrations of oridonin, 4 μmol/L T33-1/P10 in a reaction buffer of 50 mmol/L HEPES (pH 7.0), 5 mmol/L MgCl₂, 20 mmol/L NaCl was firstly incubated at 0 °C for 15 min. After that, 300 μmol/L

NTP each with or without 4 mmol/L DTT was added to the reaction buffer and incubated at 25 °C for 40 or 90 min. The reaction was quenched with equal volume of stop solution [95% (v/v) formamide, 20 mmol/L EDTA (pH 8.0), 0.02% (w/v) bromophenol blue]. The mixture was heated at 95 °C for 45 s and cooled on ice before denaturing polyacrylamide gel electrophoresis (PAGE) analysis. RNA species were resolved by 20% polyacrylamide/7 mol/L urea gel electrophoresis and then visualized by staining with Stains-All (Sigma-Aldrich). Color images obtained by scanning the stained gels were converted to gray-scale images prior to quantitation by using ImageJ (<https://imagej.nih.gov/ij>).

2.13. *In vitro* papain-like protease (PL^{pro}) inhibition assay

PL^{pro} was diluted in buffer (50 mmol/L Tris, 50 mmol/L NaCl) to 2 μmol/L and pre-incubated with different concentrations of oridonin at 25 °C for 10 min. The substrates Ub3 was diluted in buffer to a concentration of 6 μmol/L. An equal volume of K48-Ub3 substrate was then mixed with the inhibited PL^{pro} to give a final concentration of 1 μmol/L PL^{pro} and 3 μmol/L substrate. The reaction was incubated for 1 h at 25 °C. The samples were subjected to SDS gel electrophoresis and stained with Coomassie blue. Color images obtained by scanning the stained gels were converted to gray-scale images prior to quantitation by using ImageJ (<https://imagej.nih.gov/ij>).

2.14. Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software. Data were analyzed by unpaired two-tailed student's *t*-test or two-way ANOVA. n.s. not significant, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 and *****P* < 0.0001.

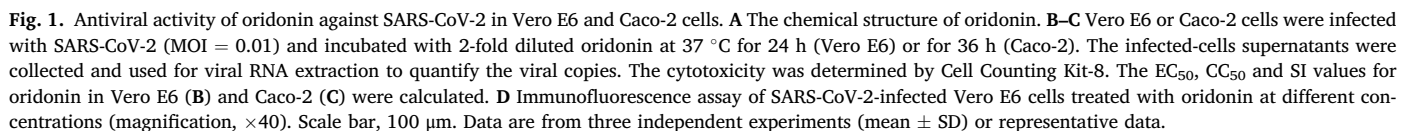
3. Results

3.1. Oridonin inhibits SARS-CoV-2 infection in different cell lines

In this study, we tested the antiviral activity of oridonin against SARS-CoV-2 infection *in vitro* (Fig. 1A). Briefly, Vero E6 cells were infected with SARS-CoV-2 (MOI = 0.01) in the presence of different concentrations of compounds. After 24 h, viral titers in cell supernatants were measured by quantitative real-time PCR (qRT-PCR). The cytotoxicity of oridonin to Vero E6 cells was evaluated by CCK8 assay. As revealed in Fig. 1B, oridonin effectively inhibited SARS-CoV-2 infection in Vero E6 cells in a dose-dependent manner with EC₅₀ and CC₅₀ values of 1.85 and >40 μmol/L, respectively, and the resulting selectivity index (SI = CC₅₀/EC₅₀) was >21.6. In addition, the oridonin also inhibited SARS-CoV-2 infection in human Caco-2 cells with EC₅₀ and CC₅₀ values of 4.12 and 25.16 μmol/L, respectively, and the resulting SI was 6.17 (Fig. 1C). Immunofluorescence assay revealed that the NP protein expression in SARS-CoV-2-infected Vero E6 cells was remarkably reduced upon treatment with oridonin (Fig. 1D). These results indicated that oridonin effectively inhibited SARS-CoV-2 infection *in vitro*.

3.2. Oridonin inhibits SARS-CoV-2 infection by reducing viral genome replication

To clarify the inhibitory steps of oridonin on SARS-CoV-2 infection, we performed the time-of-addition assay. The schematic was shown in Fig. 2A, Vero E6 cells were treated with oridonin at the following time points: full-time-infection (–1–12 h), during-time-infection (0–2 h), and post-time-infection (2–12 h). Chloroquine, an entry inhibitor of SARS-CoV-2 (Wang M. et al., 2020), and DMSO were served as controls. For all the experimental groups, cells were infected with SARS-CoV-2 at an MOI of 0.05, and the amounts of infectious viruses in culture supernatants were quantified at 12 h.p.i. by qRT-PCR assay. As indicated in Fig. 2B, oridonin significantly reduced viral RNA levels in the supernatants with full-time-infection and post-time-infection treatment.



To further investigate the inhibitory effect of oridonin on SARS-CoV-2 replication in the post-entry steps, a SARS-CoV-2 replicon containing *Renilla luciferase* (Rluc) was used for further investigation (Zhang Q. et al., 2021). Schematic of the SARS-CoV-2 replicon is shown in Fig. 3A. BHK-21 cells were electroporated with 10 µg of replicon RNA as described in Materials and Methods. The transfected cells were seeded in 12-well plate, simultaneously treated with 10 µmol/L oridonin or DMSO. Cells were collected at the indicated time points post transfection. The antiviral efficacy of oridonin against SARS-CoV-2 RNA replication was evaluated by the reduction of luciferase signals. As shown in Fig. 3B, similar to remdesivir which has been shown to inhibit the replication of SARS-CoV-2 (Wang M. et al., 2020), oridonin also effectively inhibited the SARS-CoV-2 replicon replication. These results suggested that oridonin mainly inhibits viral genome replication.

As the M^{pro} plays an essential role in viral genome replication, we therefore sought to investigate whether oridonin inhibits M^{pro} activity by

The oridonin contains α , β -unsaturated carbonyl, which could serve as a Michael receptor for targeting the thiol of cysteine residue (He et al., 2018). Recent studies have revealed that Michael acceptors, aldehydes and ketones that act as a warhead can covalently bind to the Cys145 residue in the M^{pro} pocket, rendering the protease inactive (Dai et al., 2020; Jin et al., 2020). To determine whether oridonin could covalently bind to the thiol of M^{pro}, we added the reducing agent 1, 4-dithiothreitol (DTT) in the enzymatic assay buffer in which the carbon-carbon double-bond can react covalently with DTT. As shown in Fig. 4C, in the presence of DTT, the inhibitory activity of oridonin against M^{pro} was greatly abrogated. In addition, oridonin effectively inhibited SARS-CoV-2 M^{pro} activity in a dose-dependent manner with EC₅₀ values of 13.46 μ mol/L in the absence of DTT. However, in the presence of the DTT, the EC₅₀ > 1000 μ mol/L (Fig. 4D). Similarly, the results from the transient replicon assay indicated that in the presence of DTT, viral replication capability inhibited by oridonin was recovered remarkably (Fig. 4E). Thus, we speculated that oridonin may covalently bind to the thiol of Cys145, leading to the inactivation of M^{pro} and the inhibition of viral RNA replication. Such inhibitory effect could be blunted by the reducing agent DTT.

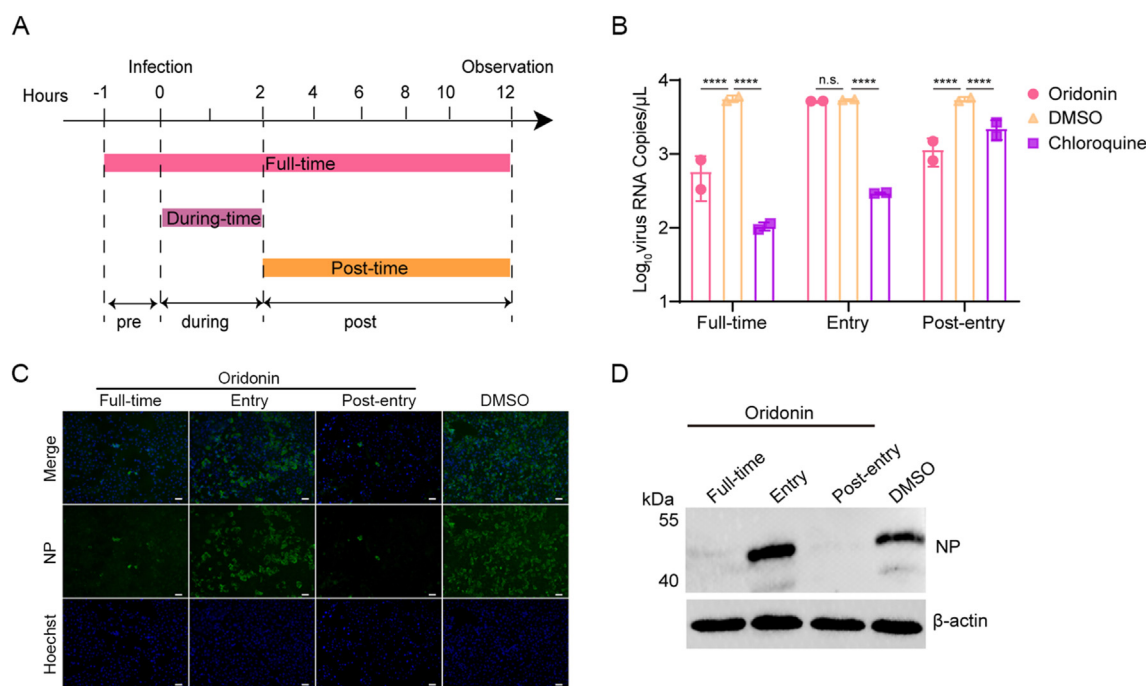


Fig. 2. Time-of-addition analysis. **A** The schematic diagram of time-of addition. Vero E6 cells were infected with SARS-CoV-2 (MOI of 0.05) at time point “0 h”. Oridonin treated the cells in the following time periods: Full-time-infection (−1–12 h), during-time-infection (0–2 h), and post-time-infection (2–12 h). Chloroquine (10 μmol/L) was used as a control. At 12 h p.i., the cell supernatants were harvested and RNA copies were detected by qRT-PCR assay (**B**). protein levels were determined by immunofluorescence assay (magnification, ×10) (**C**) and Western blotting (**D**). Scale bar, 100 μm. Data shown are representative results of two independent experiments (mean ± SD). Statistical analyses were performed using unpaired two-tailed student's *t*-test. n.s. not significant, **** $P < 0.0001$.

3.4. Oridonin displayed some inhibitory effects on viral polymerase and PL^{pro} activities at high concentrations

For coronaviruses, nsp12 RdRp and the two cofactors nsp7 and nsp8 proteins constitute the core replicase complex (nsp12-nsp7-

nsp8) in charge of the synthesis of viral RNA, playing an important role in viral replication. To investigate whether oridonin can interfere with SARS-CoV-2 polymerase activity, different concentrations of oridonin were added into the reaction of primer-dependent RNA chain extension. The RNA constructs and the expected product species were depicted in Fig. 5A. The effect of inhibitors on polymerase activity was determined by detecting the chain extension compared with the DMSO control. In the DMSO-treated group, most of the P10 can be converted to a 14-mer product (P14) within 40 min and almost fully converted at 90 min. In contrast, the addition of oridonin prevented polymerase-catalyzed chain extension in a dose dependent manner with the EC₅₀ values of 274.4 μmol/L at 40 min (Fig. 5B) and 612.2 μmol/L at 90 min (Fig. 5C), which could be attenuated by the reducing agent DTT with the EC₅₀ values of 1311 μmol/L at 40 min (Fig. 5D) and 1,124 μmol/L at 90 min (Fig. 5E). The results show that oridonin can inhibit the *in vitro* polymerase-catalyzed chain extension at high concentrations.

Similar to M^{pro}, SARS-CoV-2 PL^{pro} is also a cysteine protease with conserved catalytic triad comprising Cys, Asp, and His residues, which cleaves the N-terminal region of the polyproteins to release nsp1–3. Besides, PL^{pro} can remove ubiquitin and ISG15 from host proteins through its deubiquitinating activity, regulating virus replication and immune evasion. To determine whether oridonin affects the deubiquitinating activity of PL^{pro}, we performed *in vitro* K48-Ub3 cleavage assay catalyzed by PL^{pro}. Different concentrations of oridonin were added to the reaction system containing DTT or not. As presented in Fig. 6A, PL^{pro} hydrolyzed K48-Ub3 and resulted in measurable K48-Ub3 protein cleavage products after 1 h incubation. Compared with the DMSO group, oridonin inhibited significantly the deubiquitinating activity of PL^{pro} in a dose-dependent manner with comparable EC₅₀ values achieved in the presence or absence of DTT (611.8 μmol/L vs. 684.5 μmol/L) (Fig. 6A and B), indicating that the reducing agent, DTT might not interfere with the inhibitory effects of oridonin on the deubiquitinating activity of PL^{pro}.

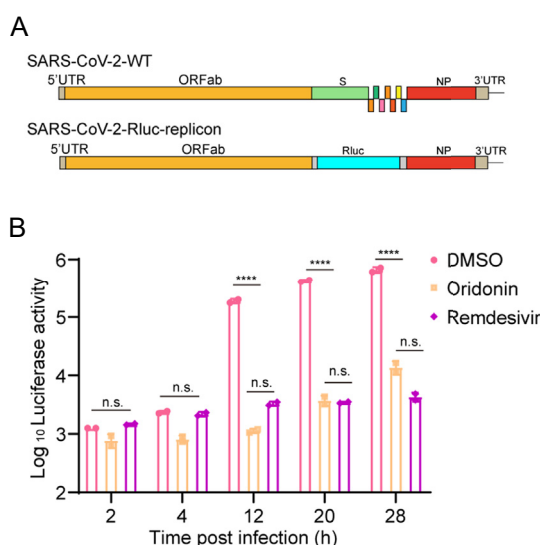


Fig. 3. Transient replicon analysis. **A** Schematic of the SARS-CoV-2 replicon. **B** Transient replicon assay. SARS-CoV-2 replicon RNA carrying a *Renilla luciferase* gene was electroporated into BHK-21 cells, followed by incubation with oridonin (10 μmol/L), Remdesivir (10 μmol/L) or DMSO at 37 °C. The cells were used for luciferase assay at different times post transfection. Data shown are representative results of two independent experiments (mean ± SD). Statistical analyses were performed using two-way ANOVA. n.s. not significant, **** $P < 0.0001$.

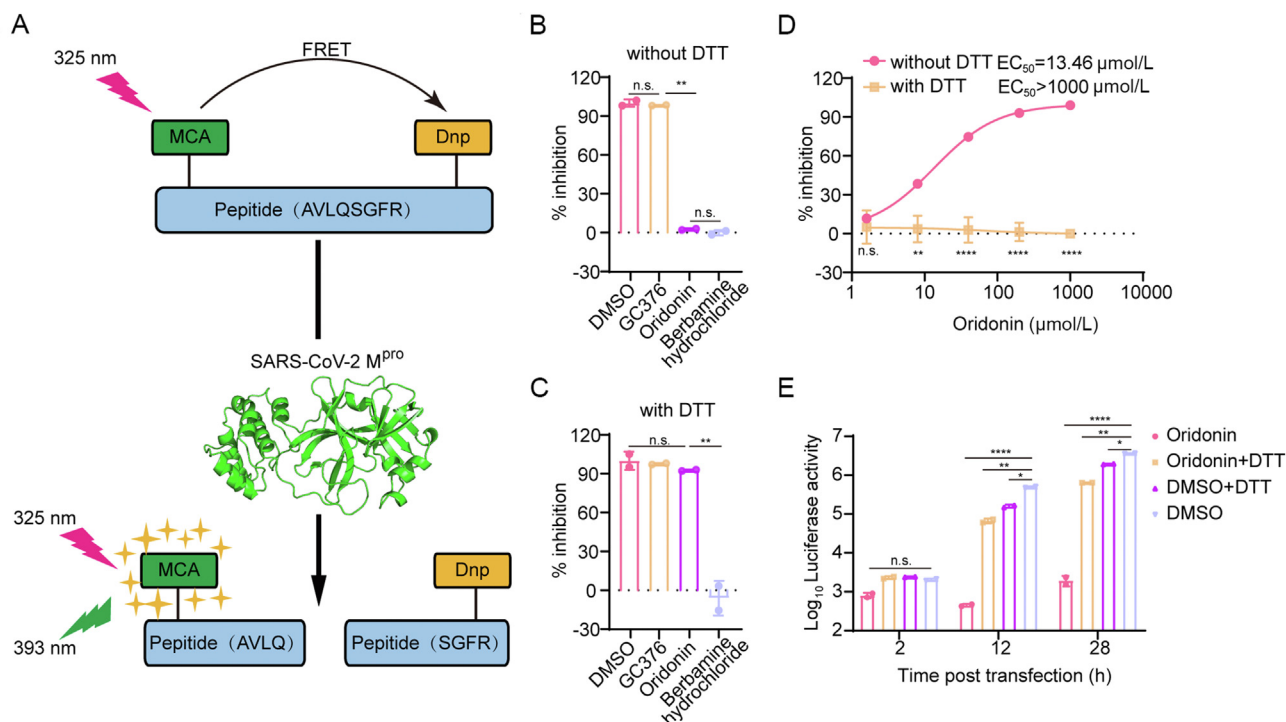


Fig. 4. Effect of DTT on the inhibition of oridonin against SARS-CoV-2 replicon and M^{pro} *in vitro*. **A** Schematic diagram of M^{pro} inhibition assay. **B–D** SARS-CoV-2 M^{pro} was incubated with positive compound GC376 (2 mmol/L), DMSO and oridonin (2 mmol/L) in the absence (**B**) or presence (**C**) of 4 mmol/L DTT at 37 °C for 10 min. Then the substrate was added to the mixture and incubated in dark at 37 °C for 5 min. Fluorescence was measured within 10 min using a multimode Microplate Reader. **D** The M^{pro} inhibitory curves of oridonin *in vitro*. SARS-CoV-2 M^{pro} was incubated with 2-fold diluted oridonin in the absence or presence of 4 mmol/L DTT at 37 °C for 10 min. Then the substrate was added to the mixture and incubated in dark at 37 °C for 5 min. Fluorescence was measured within 10 min using a multimode Microplate Reader. **E** Transient replicon assay. SARS-CoV-2 replicon RNA carrying a *Renilla luciferase* gene was electroporated into BHK-21 cells, followed by incubation with oridonin (10 μmol/L) in the absence or presence of 4 mmol/L DTT. The cells were used for luciferase assay at different times post transfection. Data shown are representative results of two independent experiments (mean ± SD). Statistical analyses were performed using unpaired two-tailed student's *t*-test or two-way ANOVA. n.s. not significant, * *P* < 0.05, ** *P* < 0.01 and **** *P* < 0.0001.

3.5. SARS-like CoV and SARS-CoV-2 Omicron variant are also highly sensitive to oridonin treatment

To test whether oridonin could be feasible to inhibit other coronavirus replication, the bat SARS-like CoV and SARS-CoV-2 Omicron variant (BA.1 and BA.2) were used. As shown in Fig. 7A, oridonin inhibited bat SARS-like CoV infection in Vero-E6 cells with the EC₅₀ values of 2.33 μmol/L. In particular, SARS-CoV-2 Omicron variant (BA.1 and BA.2) was also highly sensitive to oridonin treatment. The EC₅₀ values were 9.39 μmol/L and 6.35 μmol/L against Omicron (BA.1) and Omicron (BA.2), respectively (Fig. 7B and C). These results indicate that oridonin has broad-spectrum anti-coronavirus activity.

4. Discussion

The COVID-19 pandemic caused by SARS-CoV-2 has become the most severe threat to global health. Extensive efforts have been made to identify efficient antiviral compounds since its outbreak. And several drugs, such as Paxlovid (Owen et al., 2021), Molnupiravir (Saravolatz et al., 2023), Azvudine (Zhang J. et al., 2021) and VV116 (McCarthy, 2023) have been approved for clinical use. However, the emergence of new SARS-CoV-2 variants and virus resistance to drugs present a challenge to the development of antiviral drugs. Thus, it is still urgent to identify new candidate inhibitors to treat the SARS-CoV-2 infection. In this study, we found that oridonin could inhibit *in vitro* SARS-CoV-2 infection in the post-entry steps, mainly targeting viral replication.

Oridonin has been suggested to have the potential to react with thiol groups (Fujita et al., 1976). As shown in Fig. 1A, the oridonin contains an active α, β-unsaturated carbonyl, which has a potential to react covalently with the thiols of reducing agent via Michael addition reaction.

The reducing agent such as DTT pretreatment could reverse oridonin-induced outcomes (Huang et al., 2017; Ye et al., 2012). Here, our *in vitro* transient replicon assay and M^{pro} inhibition assay suggested that the oridonin significantly inhibited viral genome replication and the activity of M^{pro} in the absence of DTT, which could be greatly impaired by the addition of DTT. It indicates that oridonin possibly used the same strategy to exert its anti-SARS-CoV-2 activity.

Recent studies have revealed that Michael acceptors, aldehydes and ketones, which act as warhead can covalently bind to the Cys145 residue in the M^{pro} pocket and render the protease inactive (Dai et al., 2020; Jin et al., 2020). GC376, a peptide aldehydes compound, has a covalent modification on the nucleophilic Cys145 residue (Vuong et al., 2020). N3 was an irreversible inhibitor of M^{pro} with a Michael acceptor, in which the C atom of the α, β-unsaturated carbonyl forms a covalent bond with the S atom of C145 of M^{pro} via Michael addition reaction (Jin et al., 2020; Vuong et al., 2020). Therefore, we speculate that α, β-unsaturated carbonyl of oridonin, as an electrophile warhead, could covalently bind to the active Cys 145 residue of M^{pro}, and play an important role in the antiviral activities against SARS-CoV-2. Baisan Zhong et al. performed fluorescence-based thermal shift assay to screen for SARS-CoV-2 M^{pro} inhibitors and discovered that oridonin could bind to the M^{pro} catalytic site by forming a C–S covalent bond, and block substrate binding (Zhong et al., 2022). This is consistent with our results.

Such relatively broad mode of action of oridonin promoted us to further dig into its other potential targets involved in viral replication, like the key replication proteins RdRp (nsp12) and its two cofactors nsp7+nsp8, and the viral cysteine protease PL^{pro}. The anti-polymerase activity of oridonin was characterized by *in vitro* primer-dependent RNA chain extension assay. It showed that the polymerase-catalyzed

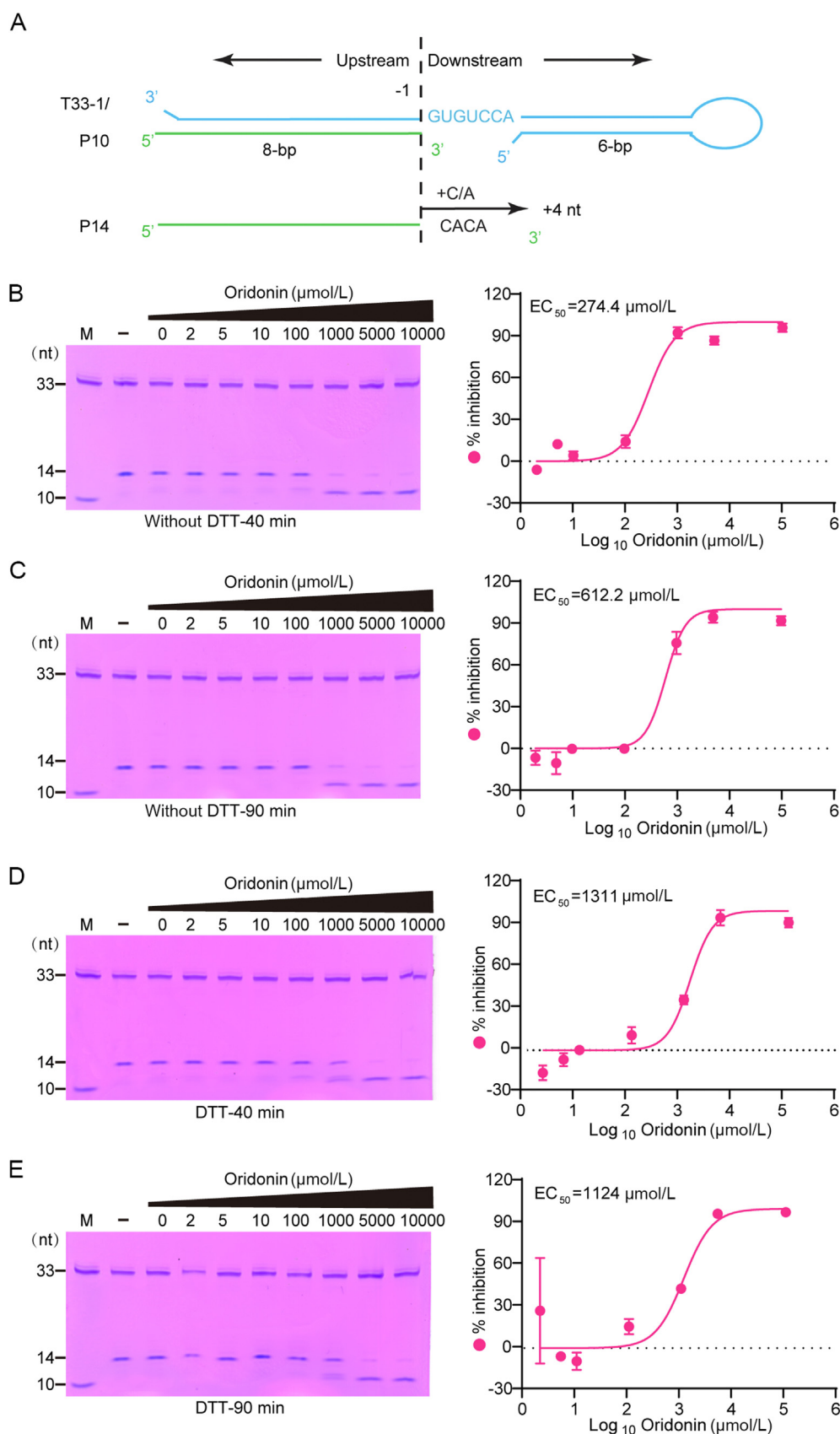


Fig. 5. Effect of oridonin on RdRp-catalyzed chain extension activity. **A** The RNA constructs and the expected product species. **B–E** RdRp, nsp7, nsp8, different concentration of oridonin, T33-1/P10 was firstly incubated at 0 °C for 15 min. Subsequently, NTP was added to the reaction buffer in the absence (**B**) or presence (**C**) of 4 mmol/L DTT and incubated at 25 °C for 40 or 90 min. The mixture was heated at 95 °C for 45 s and cooled on ice before denaturing polyacrylamide gel electrophoresis (PAGE) analysis. Data shown are representative results of two independent experiments (mean $\pm$ SD).

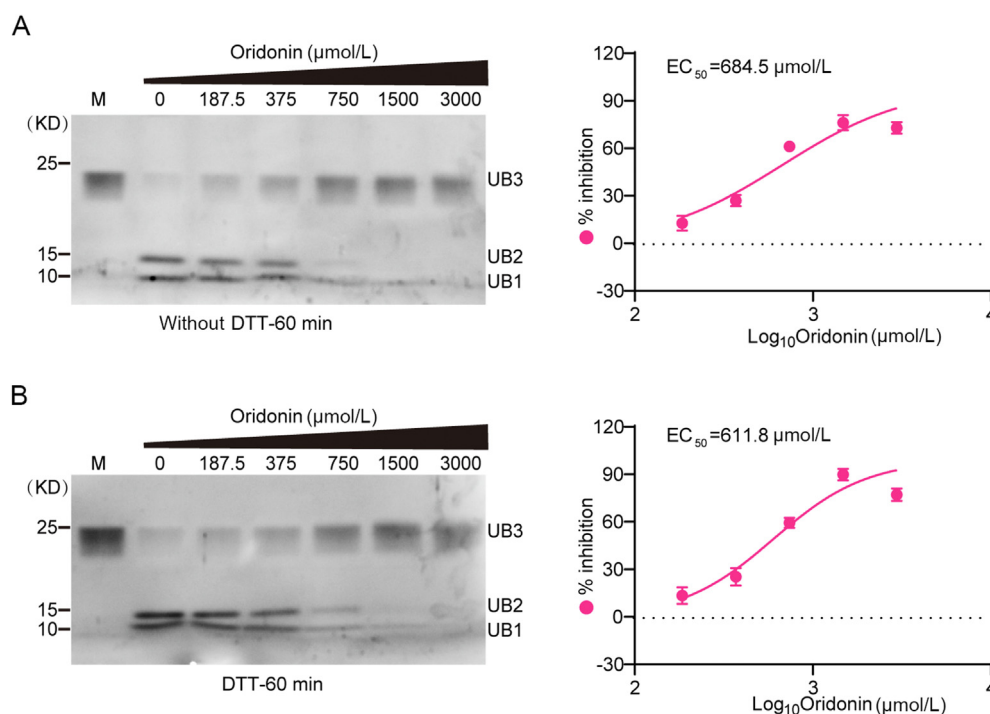


Fig. 6. Impact of oridonin on PL^{pro} deubiquitinating activity. SARS-CoV-2 PL^{pro} was incubated with different concentrations of oridonin in the absence (A) or presence (B) of 4 mmol/L DTT at 25 °C for 10 min. Then the K48-UB3 was added to the mixture and incubated at 25 °C for 1 h. After reactions were terminated, the samples were subjected to SDS gel electrophoresis and stained with Coomassie blue. Data shown are representative results of two independent experiments (mean ± SD).

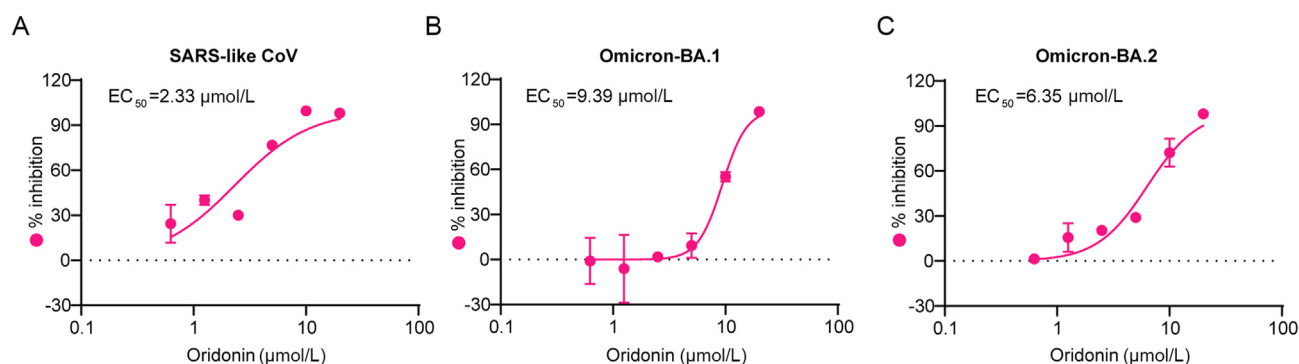


Fig. 7. Antiviral activity of oridonin against SARS-CoV-2 Omicron and bat SARS-Like CoV in Vero E6 cells. Vero E6 cells were infected at an MOI of 0.01 with bat SARS-Like CoV or SARS-CoV-2 Omicron variant (BA.1 and BA.2) and incubated with 2-fold diluted oridonin at 37 °C for 24 h (SARS-Like CoV) or for 48 h (SARS-CoV-2 Omicron variant). The cell culture fluids were harvested for plaque (A) or qRT-PCR assay (B and C). The EC₅₀ values for oridonin were shown above the figures. Data shown are representative results of two independent experiments (mean ± SD).

RNA synthesis was inhibited by oridonin only at high concentrations, and the EC₅₀ values without DTT addition were more than one log higher than those for M^{pro}. Similarly, it was found only high concentrations of oridonin had inhibitory effects on the deubiquitinating activity of PL^{pro}. It thus remains to be explored the specificity of oridonin function on these two viral elements-catalyzed events. Dene R. Littler et al. carried out a native mass-spectrometry-based approach to screen a natural product library for nsp9 binders, and identified that oridonin showed high affinity with the SARS-CoV-2 nsp9 (Littler et al., 2021). These studies suggest that other virus proteins might be potential targets of oridonin against SARS-CoV-2.

Besides directly targeting viral factors, oridonin has been shown to have strong anti-inflammatory properties by inhibition of the NLRP3 and NF-κB pathway and activation of the Keap1/Nrf2 pathway (He et al., 2018; Yang et al., 2019, 2020). There are many evidences in the literature showing that SARS-CoV-2 infection stimulates NLRP3 inflammasome activation, leading to the release of inflammatory cytokines (IL-1β,

IL-6 and TNF) and tissue inflammation (Ratajczak and Kucia, 2020; Shah, 2020). It was reported that the Keap1/Nrf2 pathway was suppressed in lung biopsies from COVID-19 patients (Cuadrado et al., 2020). The activation of the Keap1/Nrf2 pathway by Nrf2 agonists or siRNA silencing of Keap1, the inhibitor of Nrf2, decreased the host inflammatory response and inhibited the replication of SARS-CoV-2 (Olagnier et al., 2020). Therefore, it is very possible that the anti-inflammatory effects of oridonin may contribute to the inhibition of SARS-CoV-2 infection besides impairing viral replication through targeting viral proteinase and polymerase. Further investigations on the therapeutic efficacy of oridonin in tissue injury and respiratory inflammation from SARS-CoV-2 infection are still needed.

5. Conclusions

In summary, our present study shows that oridonin can effectively inhibit SARS-CoV-2 infection and replication. M^{pro} is the main target of

oridonin. This study puts forward a potential use of oridonin as a viral replication inhibitor for COVID-19 therapies.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

This article does not contain any studies with human or animal subjects performed by any of the authors.

Author contributions

Zherui Zhang: investigation, data curation, writing- original draft. Hongqing Zhang: investigation, data curation. Yanan Zhang: investigation, data curation, funding acquisition. Qiuyan Zhang: investigation, funding acquisition. Qiajie Liu: investigation. Yanyan Hu: investigation. Xiaoling Chen: investigation. Jing Wang: investigation. Yujia Shi: investigation. Chenglin Deng: investigation. Peng Gong: conceptualization, funding acquisition. Bo Zhang: conceptualization, supervision. Xiaodan Li: conceptualization, methodology, supervision. Bing Zhu: conceptualization, methodology, writing - review & editing, supervision. Hanqing Ye: conceptualization, methodology, writing - review & editing, supervision, funding acquisition.

Conflict of interest

The authors declare that they have no conflict of interest. Bo Zhang is an editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article.

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