Novel Herbal Formulation Jing Si Exhibits Multiple Functions to Inhibit Replication Activity and Subsides Viral Load of COVID-19 Variants

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Research

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Abstract

Background: SARS-CoV-2 is susceptible to frequent mutations and gets transformed into variants therefore identifying novel multi targeting remedies is necessary in formulating strategies to overcome the pandemic.

Methods: Traditional Chinese medicine based formula Jing Si herbal (JSH) was screened and analyzed by HPLC to evaluate its ability to act against infection by SARS-CoV-2 variants. The 3CL protease and RdRp assay kit were utilized to detect the enzyme activity. In order to determine the effect of JSH on the binding efficiency and viral penetration of SARS-CoV-2 variants, Calu-3 lung cells and Caco-2 colon cells were infected with fluorescent SARS-CoV-2 pseudo type lentiviruses. In addition, the effect of JSH (16.22 mg /mice/day and 48.66 mg/mice/day) on the viral load in SKH1J mice exposed to inhalation of luminescent SARS-CoV-2 variants for three days was determined.

Results: The JSH was found to be effective in inhibiting the viral entry into Calu-3 and Caco-2 cells and in mice pre-treated with JSH for 3 days also inhibited the viral load exposed to different SARS-CoV-2 variants. Interestingly, JSH also decreased 3cL and RdRp activity thereby revealing the multi targeting nature of JSH and therefore will be a potential preventive SARS-CoV-2 infection.

Conclusion: Taken together, our present results revealed that JSH could be a potential candidate for COVID-19 treatment.

1. Introduction:

The Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection pandemic has caused terribly high morbidity and mortality. While the present treatment strategies are unsatisfactory, the virus emerges stronger with different mutated variants. Moreover due to unequal access to vaccines has caused a high vulnerability to further waves of infection in the coming months and it is seen to be a danger awaiting. With sudden surge in the number of cases health infrastructure in many parts of the world is facing a break down due to over burden. While the current treatment modality is to relieve the symptoms, maintain the oxygen saturation and manage the comorbidities like hypertension and diabetes that increases the susceptibility, there are no FDA approved pharmacological drugs that target the viral infection.

In order to find a safer and effective remedy, understanding the pathophysiology of SARS-CoV-2 infection is imminent and in this regard, the mechanism of viral entry and replication is now clearly elucidated. The similarities in the infection mechanism of SARS-CoV-2 and SARS-CoV are obvious; SARS-CoV-2 like the SARS-CoV uses ACE-2 as the receptor to enter the host cell. Besides humans, other animals like pig, ferret, rhesus monkey, civet, cat, pangolin, rabbit and dog are also susceptible to SARS-CoV-2 for their compatible ACE-2 recognition[1]. To secure entry in to the host cells, the SARS-CoV-2 uses its Spike (S) protein that help in their adherence to the ACE-2 expressing cells[2]. Transmission of SARS-CoV-2 has been now widely accepted to occur through respiratory droplets. The virus is detected in the nasal Mid-

turbinate swabs implying that the nasopharynx is a site of replication of the virus. The prominent receptor, ACE2 is expressed in alveolar epithelial type-II cells[3]. However, highest level of ACE2 expression largely occurs in humans in the brush border of intestinal enterocytes. Although respiratory symptoms are the common clinical symptom of SARS-CoV-2 infection, gastrointestinal symptoms are observed prior or following the respiratory symptoms in several patients[4,5]. Recent findings show that different mutant variants of SARS-CoV-2 show different infection sites. The D614G substitution variant show enhanced potential for entry and higher replication in airways and the variant is mostly restricted with the upper respiratory tract[6]. The B.1.1.7 ( ) considered being variant of concern that gets highly accumulated in lung as well as in intestine. Another variant B.1.351 (501Y.V2, β) is 19 times less effective against vaccine induced immunity. As these mutations occur in the S-protein of the virus, they may evade drugs that target SARS-CoV-2-ACE-2 binding. Therefore, in addition to targeting S-protein binding, drugs acting on other effective targets are investigated for better efficacy.

Recent studies show that the entry of SARS-CoV2 is facilitated by TMPRSS2 protease activity[7–9]. Subsequent to ACE-2 adhesion and TMPRSS2 activity mediated membrane fusion and endocytosis, the virus gains entry and is available in the host for viral processing by hijacking the host replication machinery. The single stranded RNA of the SARS-CoV2 is then translated to an 800 KDa polypeptide (PP) that get proteolytically cleaved by the viral proteases papain like proteases (PLpro) and 3-chyomotrypsin like protease (3CLpro). RNA-dependent RNA polymerase (RdRp) synthesis the complementary RNA strand from the viral RNA template, and is a crucial enzyme for viral replication[10]. The 3CLpro and RdRp being respectively the main protease and RNA polymerase that play the major role in viral replication are suitable target for SARS-CoV2 drugs[11–14]. In this regard, Traditional Chinese Medicine (TCM) gains attention for its important role in the treatment of diseases via its multicomponent, multitarget, and multipathway approach. In this study, a Traditional Chinese Medicine based formula Jing Si has been identified for its anti- SARS-CoV2 infection effects. Jing Si is a combination of eight herbs of Chinese pharmacopoeia that are also listed in Taiwan herbal pharmacopeia for their blood homeostasis value, antiviral effect, inflammatory effect, beneficial effects against lung infection and to treat outcomes like dry cough, blood tinged sputum etc. The herbal tea “Jing Shi” was tested for their ability to inhibit infection by different SARS-CoV2 variants and for their potential to target SARS-CoV2 3CLpro and RdRp involved in viral replication.

2. Material And Methods:

2.1.Materials and reference compounds:

Jing Si herbal tea was obtained from the Chinese Medicine Department of Hualien Tzu Chi Hospital, Taiwan. 3’,4’-Dimethoxyflavone, Swertisin, Harmine, Nerolidol and Eupatilin were purchased from sigma-Aldrich (St. Louis, MO, United States). DMEM and FBS were procured from Gibco, Thermo Fisher Scientific (Waltham, Massachusetts, United States). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Thermo Fisher Scientific.

2.2.Preparation of Jin Si herbal tea (JSH)

The JSH pack containing of leaves of *Artemisia argyi* (6 g), *Ohwia caudate* (6 g), *Ophiopogon japonicas* (4 g, Ophiopogonis Radix) and *Perilla frutescens* (2 g), Roots of *Houttuynia cordata* (4 g), *Platycodon grandifloras* (4 g) and *Glycyrrhiza uralensis* (2 g, *Glycyrrhizae radix*); and flowers of *Chrysanthemum × morifolium* (0.2 g) were boiled with 600 mL RO water and concentrated to 60 mL.

2.3.Cell culture

Human colorectal grade II adenocarcinoma cells Caco-2 cells and human lung grade I adenocarcinoma Calu-3 cells were cultured in Eagle's Minimum Essential Medium containing 20% serum. The cells were cultures in a CO_2 incubator at 37°C with 95% Air, 5% CO_2. The viral infection assay was performed on (2 x 10^3 Caco-2 (Colon) and Calu-3 (lung) fluorescent SARS-CoV-2-S protein pseudo type lentiviruses (10 MOI).

2.4.Measurement of RNA dependent RNA polymerase activity.

The measurement of the RNA molecules synthesized by the RNA polymerase and the effect of JSH on the polymerase activity was performed using Viral (Flavivirus) RNA-dependent RNA Polymerase Assay Kit (ProFoldin, Hudson, MA, USA) following manufacturer's protocol. Briefly, the reaction volume consisting of reaction mixture is 30 µL including 23.1 µL of H_2O, 3 µL of 10 x Buffer, 3 µL of 10 x $MnCl_2$, 0.3 µL of 100 x template, 0.3 µL of 100 x RNA polymerase and 0.3 µL of 100 x NTPs were incubated at 37°C for 60 min. After incubation, 30 µl of the 1 x fluorescence dye was added and the fluorescence intensity was measured at 535 nm.

2.5.Quantification of 3cL Protease activity.

The protease activity of 3cL protease to cleave fluorescence FRET peptide substrate was measure using SensoLyte® 520 SARS-CoV-2 3CL Protease Activity Assay Kit following manufacturer's methods. Briefly, 10 µL of JSH was added to 40 µL of enzyme solution in a 96 well plate. In separate wells, positive control, inhibitor control, vehicle control, test compound control, substrate control was taken. To the content in the well 50 µL of 3cL protease was added and mixed for 30 seconds and incubated for 30 minutes. The activity was measured at 490 nm/520 nm.

2.6. In vitro GFP-Pseudovirus infection and observation

The SARS-CoV-2 pseudotyped lentivirus bearing green fluorescent protein (GFP) were obtained from National RNAi Core Facility at Academia Sinica conducted to Caco-2 or Calu-3 cells with 10 MOI after

treated with 10 µg/ml and 30 µg/ml JSH for 16 hours. After 48 hours, the infected cells with GFP were captured under fluorescent microscope and quantified by detector (BioTek, Agilent, HTX, Taoyuan, Taiwan). Data processing and quantification were performed with Gen5 software.

2.7. Animal experiments:

All the experiments were performed after prior approval from the Institutional Animal care and ethics committee of The Tzu Chi hospital. Eight weeks old SKH1/J mice were used to ascertain the effect of JSH on viral adhesion and infective viral load of different variants of SARS-CoV-2. The animals were grouped (n=7) into control, SARS-CoV-2 D614G, SARS-CoV-2 B.1.1.7, SARS-CoV-2 B.1.351 (501Y.V2), D614G dose 1 JSH, D614G dose 2 JSH, B.1.1.7 dose 1 JSH, B.1.1.7 dose 2 JSH, B.1.351 (501Y.V2) dose 1 JSH, B.1.351 (501Y.V2) dose 2 JSH. The treatment mice were pre-treated with two (16.22 mg /mice/day and 48.66 mg/mice/day) different doses of JSH for three days. From the fourth day, in addition to JSH administration the mice in the treatment group and the SARS-CoV2 were nasally administered with 500 µL of 1.2 X 10^6 luminescent viral particles every day for three continues days using a nebulizer (Aeroneb USB controller, Kent Scientific Corporation Torrington, CT, USA) with a flow rate of 0.4 mL/min. On the seventh day the viral load accumulated in the mice was determined by imaging using an In Vivo Imaging System (IVIS, PerkinElmer, UK).

2.8.Statistical analysis

Data are calculated as the mean ± SD and statistical significance was assessed by SigmaPlot software version 10.0 (Systat Software Inc., San Jose, CA, USA)

3. Results:

3.1. Identification and characterization of JSH

Jing Si is a combination of eight common herbs of Chinese pharmacopoeia including *Artemisia argyi*, *Ohwia caudate*, *Ophiopogon japonicas*, *Perilla frutescens*, Houttuynia cordata, *Platycodon grandifloras*, *Glycyrrhiza uralensis* and *Chrysanthemum × morifolium.* The characterization of JSH was performed by HPLC MS/MS analysis. The results indicated that Swertisin and Eupatilin were two major components from *Artemisia argyi* and *Ohwia caudate* which were abundant in JSH (Sup Fig. 1). Eupatilin is an O-methylated flavone could be isolated from *Artemisia argyi* with antioxidant capacity[15]. Swertisin has been found can against Influenza and hepatitis B virus[16]. It was found that Swertisin can contribute to inhibit replication by docking analysis for SARS-CoV2 infection which binding to RNA-dependent RNA polymerase (RdRp)[17].

3.2 Inhibition of 3CL protease and RNA dependent RNA polymerase to inactivate the initiation of viral replication

machinery

To reveal the effects of viral replication, we examined and found that JSH reduced the activity of the 3CL protease by 35-40% (Figure 1A). In addition, JSH also suppressed the activity of RNA dependent RNA polymerase from 50 mg/ml to 300 mg/ml in a dose dependent manner (Figure 1B). The results demonstrated that JSH might have the ability to inhibit the viral replication

3.3. Effect of JSH on multiple mutant variants infection on Colon cells

In order to find the effect of JSH in inhibiting infection, Caco-2 colon cells infected with Wild type, D614G, B1.1.7, 501Y.V2, B.1.429 and B.1.617.2 SARS-CoV-2-S pseudo type GFP-lentiviruses (MOI=10) were analyzed for the viral entry after transduction. The results showed that JSH reduces the infection in Caco-2 cells by 51-80% as examined from the reduced fluorescence intensity (Figure 2A & B).

3.4. Effect of JSH on multiple mutant virus strains infection on lung cells

The effect of JSH in inhibiting lower respiratory tract infection was determined using ACE-2 rich Calu-3 cells that were infected with Wild type, D614G, B.1.1.7 and 501Y.V2 SARS-CoV-2-S-protein pseudotype lentiviruses and were then analyzed for the viral entry by measuring the fluorescence intensity. JSH inhibited the infection in Calu-3 cells by 64-86% as seen under fluorescent microscope and quantified by the reader (Figure 3A & B). These results pointed out that JSH could inhibit SARS-CoV-2-S-protein pseudotype lentiviruses infection on ACE2 abundant cells.

3.5. JSH inhibits infection by SARS-CoV-2 variants of concerne :

From the in vitro study we found that JSH could prevent pseudotype lentiviruses infection. We utilized the transduction of SARS-CoV-2-S-protein pseudotype lentiviruses bearing luminescence in animal model to test the effort. IVIS imaging of the SARS-CoV2 infected animals showed that variants (D614G, B.1.1.7, N501Y.V2, B.1.617.2) luminescent pseudotype lentiviruses accumulated highly in the upper respiratory tract and the lower digestive tract but pre-treating JSH effectively decreased the viral load. The results therefore showed that JSH administration limits the infection severity and might rescue the succumbing patients with different variant from lung infection by reducing the infection intensity to moderate or mild levels.

4. Discussion:

Remdesivir is the only FDA-approved drug for the treatment of COVID-19 patients. Remdesivir, a phosphoramidate prodrug that metabolizes to adenosine C-nucleoside triphosphate in the respiratory epithelial cells, act as nucleoside analog and hinder the function of RNA-dependent RNA polymerase

(RdRp) to inhibit SARS-CoV-2 replication[18,19]. Molecular docking studies reaveal that Remdesivir also interacts with 3cL protease contributing to its effectiveness in treating COVID-19[20]. However, clinical studies have so far given mixed results with no conclusive evidence and few known side effects[21,22]. Generation of variants of SARS-CoV-2 has increased the concerns on the effectiveness of the drugs and even the vaccines[23]. The many variants like D614G, B.1.1.7 and show enhanced viral replication in human lung and airway tissues[24–27]. Causing rapid onset of the immediate pulmonary effects and reduced oxygen saturation in the patients. Variants like B.1.351 showed higher resistant to antibodies and vaccination[28]. The potentials effect of JSH will be of appreciable benefit as they are effective against all the variants in concerns.

In our studies JSH has been found to effectively inhibit 3cL and RdRp activity to render anti- SARS-CoV-2 infection effects. Moreover, the animal studies point out that oral JSH administration effectively suppresses both variants that infest the respiratory tract as well as those in the gastrointestinal tract. Therefore JSH may display a pleiotropic in order to suppress several variants of SARS-CoV-2, and wider consumption of JSH or its components among population may also benefit in reducing the generation of new variants.

The constituents of JSH have shown promising evidences for antiviral effects that forms the rational for the antiviral preparation. The flavonoids in *Ohwia caudate* provide antiviral activity against viruses like influenza[16]. Swertisin, a flavonoid known for its antiviral activity against Anti-hepatitis B and Influenza a Virus is also known to inhibit SARS-CoV2 RdRp in in silico analysis [29]. In addition, Isoliquiritigenin, an active metabolite of *Glycyrrhiza uralensis* has been previously shown to inhibit Influenza A, hepatitis C in in vitro as well as in in vivo conditions. *Ophiopogon japonicas*, consumed as a functional food in China for a long period of time contain polysaccharides that is cardio-protective in conditions like diabetes[30]. The *O. japonicus* polysaccharides is potential component of JSH in controlling the comorbidities like diabetes and hypertension associated cardiovascular disease are the major risk factors of SARS-CoV2 infection [31]. *Houttuynia cordata* is one of the components in the TCM based formula used in China to manage SARS-CoV outbreak[32]. It exhibited significant inhibitory effects on SARS-CoV 3C-like protease (3CL) and RNA-dependent RNA polymerase (RdRp). Platycodin D from *Platycodon grandiflorum*, has been recently identified to prevent SARS-CoV-2 infection via inhibition of lysosome- and TMPRSS2-driven SARS-CoV-2-entry by disrupting the host-cell membrane cholesterol[33]. Leaf extracts of *Perilla frutescens* prevents SARS-CoV-2 viral entry into host cells by inactivating the virus and showed a synergetic improvement when treated in combination with remdesivir[34]. Preclinical animal studies on *Chrysanthemum morifolium* has proven their efficiency in treating lipopolysaccharide induced acute lung injury in mice due to their ability to balance levels of pro-inflammatory and anti-inflammatory factors, and inhibition of free radicals generation[35]. Therefore the constituents of JSH provide it with necessary biological factors necessary for its effects against SARS-CoV-2 infection.

Various antiviral drugs to treat COVID-19 has so far showed mixed out comes and the generation of different variants has caused concerns on the treatment efficiency.

5. Conclusion:

In our study, JSH has shown effective inhibition of viral replication among various variants which would greatly benefit in containing the spread of such variants among the masses and therefore is a potential candidate for COVID-19 treatment.

Abbreviations

3cL, 3-chyomotrypsin like protease

ACE-2, angiotensin-converting enzyme 2

COVID-19, coronavirus disease 2019

FRET, fluorescence resonance energy transfer

GFP, green fluorescent protein

JSH, Jing Si herbal tea

RdRp, RNA-dependent RNA polymerase

S-protein, spike protein

TMPRSS2, host transmembrane serine protease 2

Declarations

Availability of data and materials

Data and materials are available on request.

Conflicts of Interest

All authors declare that there are no conflict of interest to disclose.

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Author contributions

Y-J, L., T-J, H., C-Y,H., M.A.S provided the conceptualization and designed the experiments. W-W, K. curated and interpreted all the data; K.T.P.D., S-H.S. and B-Y.L. generated the figures. Y-J, L., M.A.S. wrote the main manuscript text. S-Z.L., Y-C,C reviewed and edited the manuscript. All authors analyzed and discussed all the data together.

Ethics declarations

The animal experiments in this study were approved by the Tzu Chi Hospital Animal Care and Use Committee.

Consent for publication

Not applicable.

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Figures

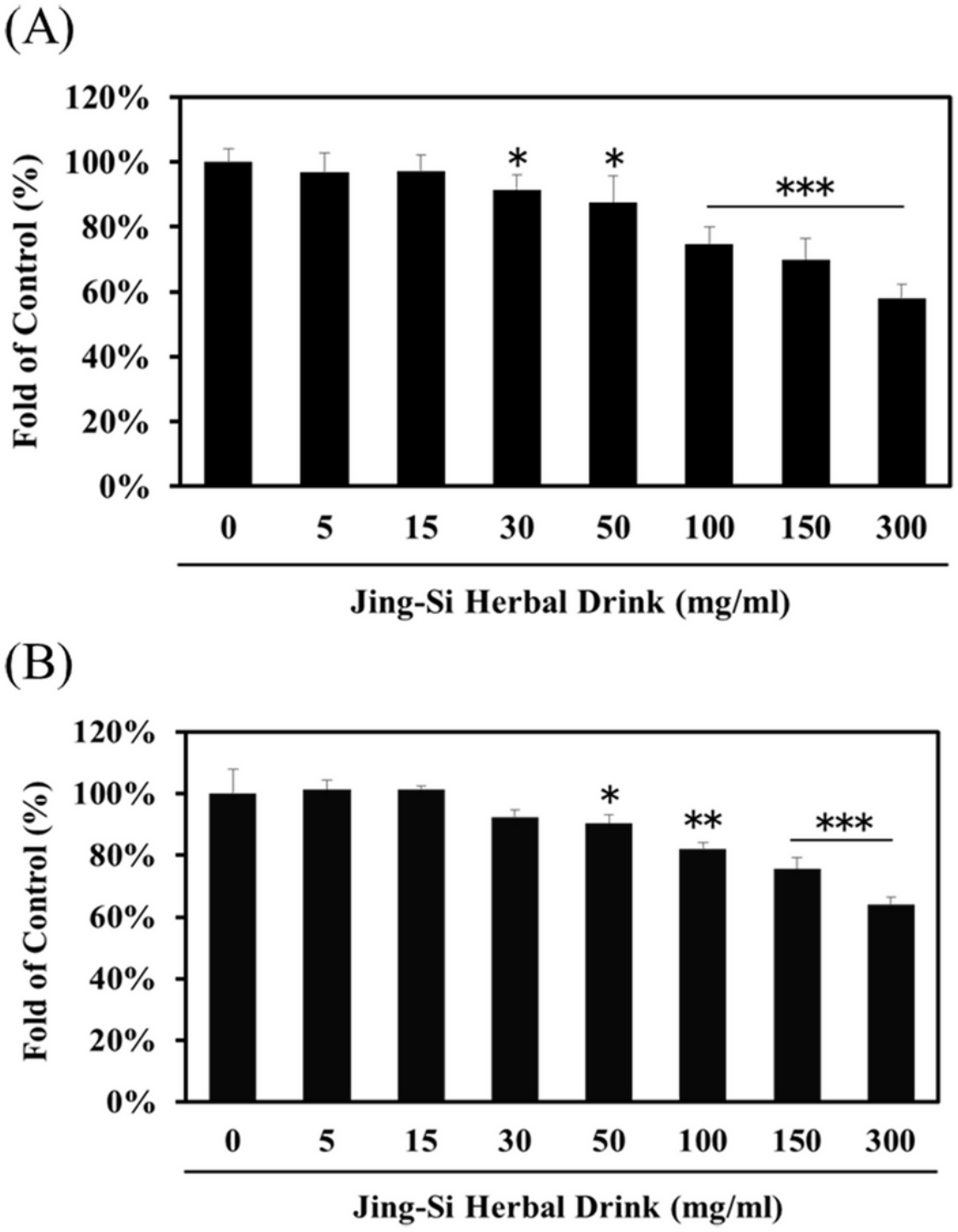

Figure 1

JSH decreased the activity of 3CL protease and RdRp in a dose dependent manner. (A) JSH with different dosages were tested the inhibitory activity of 3CL protease. The result showed that 30 mg/ml to 300 mg/ml of JSH reduced the 3CL protease activity. (B) JSH decreased the activity of RdRp from 50 mg/ml to 300 mg/ml. The data are presented as mean ± standard deviation (n = 3). Significance ascribed as *p <0.05, **p <0.01

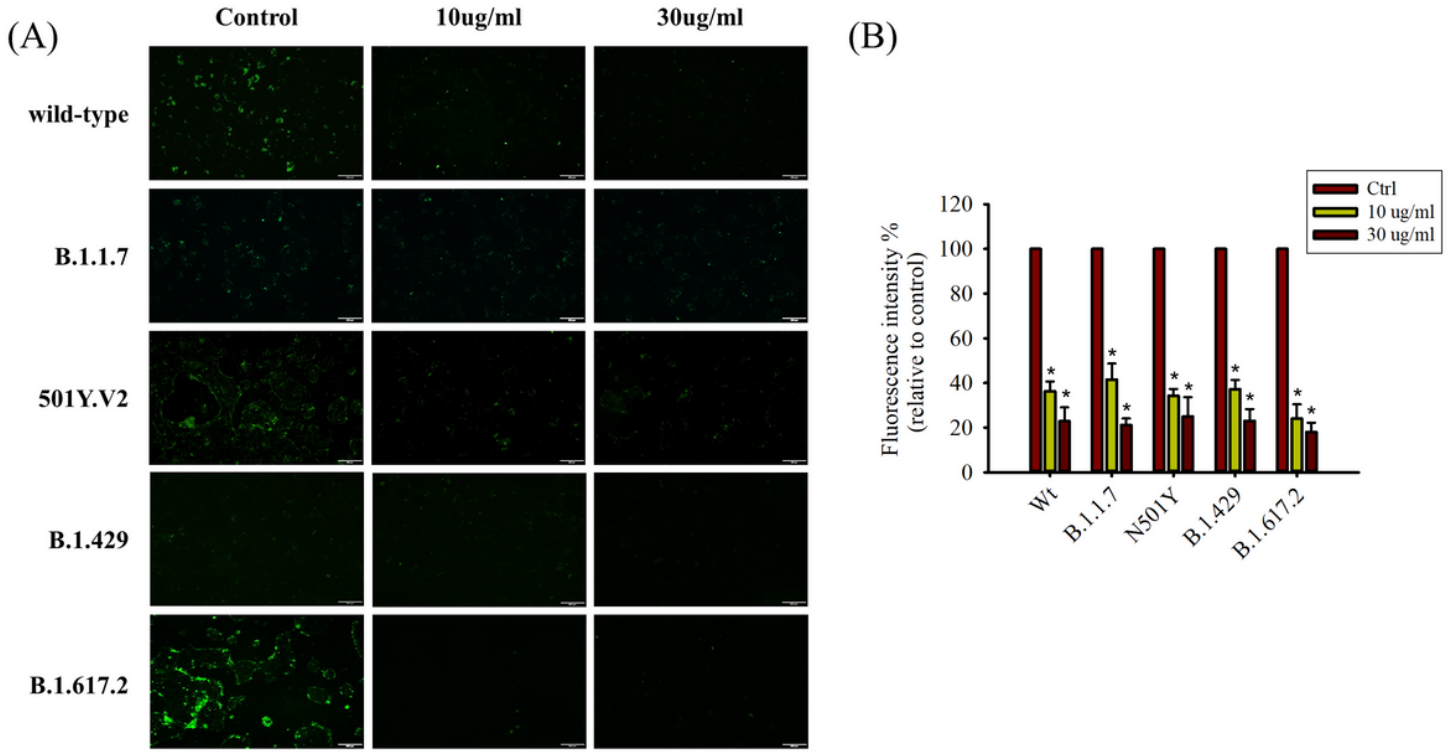

Figure 2

Effect of JSH on mutant variants infection in Caco-2 colon cancer cells.

Wild type, D614G, B1.1.7, 501Y.V2, B.1.429 and B.1.617.2 SARS-CoV-2 pseudotype lentiviruses bring GFP were conducted to Caco-2 cells with 10 MOI after treated with 10 μg/ml and 30 μg/ml JSH for 16 hours. (A) The data showed low level of transduction efficiency in different mutant variants which were under JSH pre-treatment. (B) The fluorescent intensity was detected by Synergy™ HTX. *p <0.05

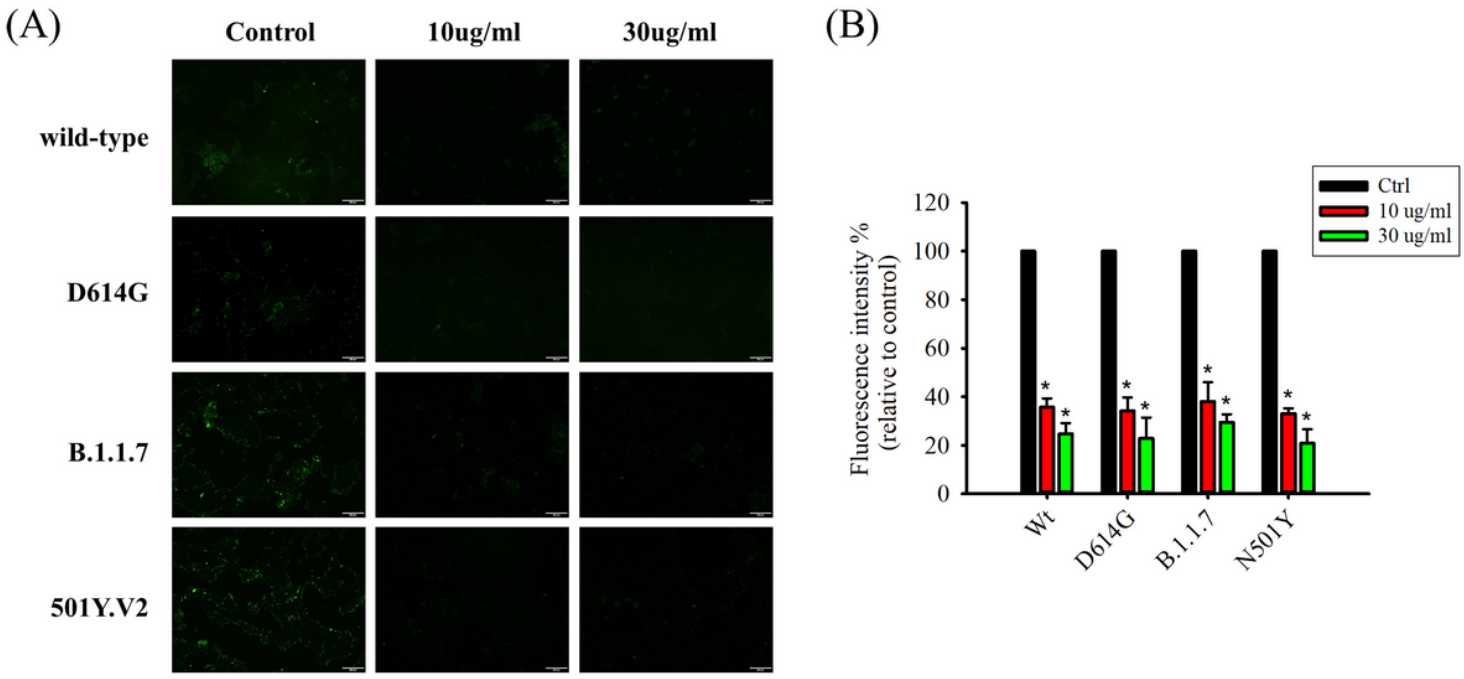

Figure 3

Effect of JSH on mutant variants infection in Calu-3 lung cancer cells.

Wild type, D614G, B1.1.7 and 501Y.V2 SARS-CoV-2 pseudotype lentiviruses bring GFP were infected with Calu-3 lung tumor cells with 10 MOI after treated with 10 μg/ml and 30 μg/ml JSH for 16 hours. (A) The data showed dramatically low level of transduction efficiency in different mutant variants which were under JSH pre-treatment compared to control. (B) The fluorescent intensity was detected by Synergy™ HTX. *p <0.05

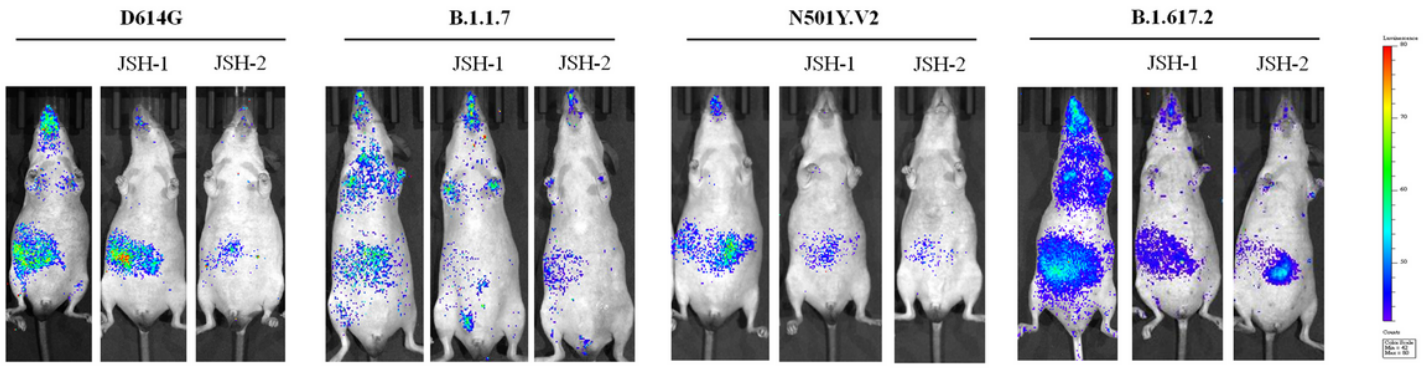

Figure 4

Effect of JSH in reducing the viral load of different variants of SARS-CoV-2. IVIS imaging of the administered fluorescent pseudo type viral particle containing different variants of S-protein (D614G, B.1.1.7, N501Y.V2, B.1.617.2) show the binding affinity of SARS-CoV2 pseudotype lentiviruses under JSH pre-treatment (JSH-1: 16.22 mg/mice/day, JSH-2: 48.66 mg/mice/day) in modulating the affinity.

Supplementary Files

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- sup.1.tif