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Original Article

Anemoside B4 inhibits SARS-CoV-2 replication *in vitro* and *in vivo*

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ABSTRACT

Objective: Anemoside B4 (AB4), the most abundant triterpenoidal saponin isolated from *Pulsatilla chinensis*, inhibited influenza virus FM1 or *Klebsiella pneumoniae*-induced pneumonia. However, the anti-SARS-CoV-2 effect of AB4 has not been unraveled. Therefore, this study aimed to determine the antiviral activity and potential mechanism of AB4 in inhibiting human coronavirus SARS-CoV-2 *in vivo* and *in vitro*.
Methods: The cytotoxicity of AB4 was evaluated using the Cell Counting Kit-8 (CCK8) assay. SARS-CoV-2 infected HEK293T, HPAEpiC, and Vero E6 cells were used for *in vitro* assays. The antiviral effect of AB4 *in vivo* was evaluated by SARS-CoV-2-infected hACE2-IRES-luc transgenic mouse model. Furthermore, label-free quantitative proteomics and bioinformatic analysis were performed to explore the potential antiviral mechanism of action of AB4. Type I IFN signaling-associated proteins were assessed using Western blotting or immumohistochemical staining.
Results: The data showed that AB4 reduced the propagation of SARS-CoV-2 along with the decreased Nucleocapsid protein (N), Spike protein (S), and 3C-like protease (3CLpro) in HEK293T cells. *In vivo* antiviral activity data revealed that AB4 inhibited viral replication and relieved pneumonia in a SARS-CoV-2 infected mouse model. We further disclosed that the antiviral activity of AB4 was associated with the enhanced interferon (IFN)-β response *via* the activation of retinoic acid-inducible gene I (RIG-1) like receptor (RLP) pathways. Additionally, label-free quantitative proteomic analyses discovered that 17 proteins were significantly altered by AB4 in the SARS-CoV-2 coronavirus infections cells. These proteins mainly clustered in RNA metabolism.
Conclusion: Our results indicated that AB4 inhibited SARS-CoV-2 replication through the RLR pathways and moderated the RNA metabolism, suggesting that it would be a potential lead compound for the development of anti-SARS-CoV-2 drugs.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by a novel coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV-2), continues to pose a global threat to human beings. SARS-CoV-2, which belongs to the family of Coronaviridae, is capable of infecting a wide variety of hosts (V'Kovski et al., 2021). Upon entry into the host cell cytoplasm, the viral genome is translated into roughly 30 proteins. Of these, 16 are initially translated as two polyproteins that must be cleaved into the individual viral proteins for infection to proceed. This cleavage is mediated by two virally encoded proteases: the main viral protease, known as Mpro, 3CLpro, or nonstructural protein 5 (nsp5); and a second protease known as the papain-like protease (PLpro), a domain within nsp3 (Chen et al., 2020). Drugs for inhibiting virus replication through inhibited protease have become a hot spot of development.

Several anti-viral drugs have been used to suppress the SARS-CoV-2 infection including remdesivir, penciclovir, lopinavir/ritonavir, and chloroquine (Wang et al., 2020). Although specific antiviral treatments are available for coronavirus infections, a lack of

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specific drugs and vaccines against the new variants of SARS-CoV-2 has resulted in a high mortality rate. In addition, some of them have serious side effects. The shortcomings of current medicines lead scientists and physicians to continuously look up for better remedies for SARS-CoV-2 infection.

Recent studies have highlighted the potential use of Chinese traditional medicine for the treatment of COVID-19 including Qingfei Paidu Decoction, Xuanfei Baidu Granules, Huashi Baidu Granules (Lyu et al., 2021; Jia, Li, Liu, Gu, & Li, 2023). *Pulsatilla chinensis* (Bunge) Regel, a popular traditional Chinese medicine, has been widely used in China for intestinal amebiasis, malaria, bacterial infections, and vaginal trichomoniasis (Luan et al., 2021). Anomoside B4 (AB4), isolated from *P. chinensis*, has anti-inflammation, anti-oxidant, anti-apoptosis, analgesia, and immunological adjuvant activities. Furthermore, reports show that AB4 has anti-virus potential activity. AB4 inhibits the secretion of IL-10 from the porcine iliac artery endothelial line challenged with porcine circoviruses (PCV2) *in vitro* and *in vivo* (Hu et al., 2016). AB4 inhibits pneumonia induced by *Klebsiella pneumoniae* and influenza virus FM1 in mice (He et al., 2020; Yuan et al., 2020). Except for these reports, our latest research shows that AB4 increases the viability of EV71-infected RD cells and suppressed EV71 replication in suckling mice infected with EV71 (Kang et al., 2022). However, the antiviral activity of AB4 on SARS-CoV-2 infection has not been investigated.

In the present study, we conducted a series of experiments to demonstrate the antiviral activities of AB4 against SARS-CoV-2 *in vitro* and *in vivo*. The antiviral activities of AB4 against SARS-CoV-2 were first assessed in HPAEpiC cells (human pulmonary alveolar epithelial cells) and HEK293T cells (human embryonic kidney cells) using CCK-8 and Western blotting assay. Then we investigated the effect of AB4 on SARS-CoV-2 infected hACE2-IRES-luc transgenic mice. We further searched for the differentially regulated proteins altered by AB4 in cell lysis samples by quantitative proteomics and analyzed the biological processes that these changed proteins participated in through the Metascape database. Our work suggest that AB4 could be used as a safe and effective therapeutic agent for treating SARS-CoV-2 coronavirus infections and may also be used as a lead molecule for novel drug development.

2. Materials and methods

2.1. Compounds

The extraction, isolation, and characterization of AB4 were processed in our lab similar to the previous publication (He et al., 2019). Briefly, AB4 (580 g, 99.5%) was extracted and obtained from the dried roots of *P. chinensis* (10 kg) using gradient alcohol solution and AB-8 macroporous resin column.

2.2. Viruses and cells

The SARS-CoV-2 (strain 107) was provided by Guangdong Provincial Center for Disease Control and Prevention (Guangzhou, China). This virus was propagated and titrated on Vero E6 cells (ATCC). Vero E6 and HEK293T (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) cells were grown in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% (volume percentage) fetal bovine serum (Gibco) and 100 U/mL penicillin–streptomycin (Gibco). HPAEpiC cells were purchased from the ScienCell Research Laboratory (San Diego, CA), and cultured according to the manufacturers instruction. All cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. All the infection experiments were performed at biosafety level-3 (BSL3) conditions at the Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences, Kunming Institute of Zoology (Kunming, China).

2.3. Western blotting and antibodies

Western blotting analysis of cell lysates were performed as previously described (Zha et al., 2023). SARS-CoV-2 N Protein antibody (Cat# A20142; 1:1 000), SARS-CoV-2 3CLpro antibody (Cat# A20198; 1:1 000), SARS-CoV-2 Spike antibody (Cat# A20497; 1:1 500), Hsp90α/β antibody (Cat# A5027; 1:1 000), TBK1 antibody (Cat# A3458; 1:1 000), phospho-IRF3-S386 antibody (Cat# AP0995; 1:1 000) and ISG15 antibody (Cat# A2416; 1:1 000) were provided by ABclonal (Wuhan, China). IFNβ antibody (Cat# A5526; 1:1 000) was purchased from Selleck (Houston, TX, USA). MAVS antibody (Cat# sc-166583; 1:1 000) was provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRF3 antibody (Cat# 66670–1-Ig; 1:1 000) was obtained from Proteintech (Wuhan, China). IFNAR1 antibody (Cat# ab45172; 1:1 000) was purchased from Abcam (Cambridge, MA, USA). β-actin antibody (Cat# AF0003; 1:1 000), HRP-labeled goat anti-mouse IgG(H + L) (Cat# A0216; 1:5 000) and HRP-labeled goat anti-rabbit IgG(H + L) (Cat# A0208; 1:5 000) were obtained from Beyotime (Shanghai, China).

2.4. Cellular antiviral activity assay

This assay was performed as previously described using CCK-8 and RT-qPCR methods (Qiao et al., 2021). Briefly, HPAEpiC or HEK293T cells were pretreated with different concentrations of AB4 (0–200 μmol/L) 4 h before virus infection. SARS-CoV-2 at a multiplicity of infection (MOI) of 1 or 0.1 was used to infect cells. The cytopathic effect caused by SARS-CoV-2 infection was quantitatively analyzed 72 h after infection of the virus using CCK-8 according to the manufacturer's protocol. Quantitative mRNA expression was assessed by real-time PCR (Bio-Rad) using the Thunderbird® Probe One-Step qRT-PCR Kit (Toyobo).

2.5. In vivo antiviral studies of AB4

The *in vivo* antiviral studies were carried out in an animal biosafety level 3 (ABSL3) facility using high-efficiency particulate air (HEPA) filtered isolators. All animal experiments were carried out according to the guidelines of the International Standards for the Care and Use of Laboratory Animals and approved by the Committee of Use of Laboratory Animals, Navy Military Medical University (Approval ID: ABSL3-003).

The *in vivo* protection efficacy of AB4 was assessed by using hACE2-IRES-luc transgenic mouse model (Shanghai Model Organisms Center, Inc.). Male hACE2-IRES-luc transgenic mice (6–8 week old) were randomly divided into three groups and infected intranasally with a dose of 1×10^4 PFU SARS-CoV-2 virus (Pubmed No: MT627325). AB4 (2.5 or 5 mg/kg) or PBS control were injected intraperitoneally once a day for 4 consecutive days. The conditions of mice including body weight and body temperature were monitored daily until the experiment was finished. Lung tissues (n = 5, per dpi group) were collected to analyze the virus burden in the lung. Virus titers are demonstrated as RNA copies per microgram tissue. RNA was extracted from lung tissues using the TRIzol™ Reagent (Invitrogen) according to the manufacturer's instructions. The remaining lung samples were fixed in 4% paraformaldehyde for histological examination.

2.6. Label-free quantitative proteomics

The detailed methods for protein sample preparation, MS analysis, database search, and protein quantification were described previously (Zhang et al., 2021). Briefly, protein samples were

separated on SDS-PAGE and stained with silver nitrate. Gel slices were excised, washed, destained, and dried. Proteins were reduced, alkylated, and digested by trypsin in gel. The resulting peptides were extracted from gel, vacuum dried, resuspended in 0.1% trifluoroacetic acid, desalted with C18 ZipTips, and then dissolved in 0.1% formic acid. The extracted peptides was analyzed on an Orbitrap Elite hybrid mass spectrometer (MS) with an electrospray ionization inlet (Thermo Fisher, Waltham, MA, USA). The MS/MS raw files were searched with Proteome Discoverer (version 2.1, Thermo Fisher Scientific) against the Human UniProt database (https://www.uniprot.org) with concatenated reverse protein sequences and common contaminants. The 1% FDR at both peptide and protein levels was applied for the analysis. Proteins were quantified with the label-free quantification included in Proteome Discoverer. The *P*-value was calculated by performing a two-sample Student's *t*-test. Log_2 (Fold Change) (Model/200 μmol/L AB4) and -Log_{10} (*P*-value) from three biological replicates were used to construct the volcano plots in Graph Prism (version 8.3.0).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.3 (GraphPad Software Ltd., San Diego, CA, USA), with the threshold for significance set at $P < 0.05$. Student's two-tailed *t*-test was used for comparison between two groups. Statistically significant differences for more than two groups were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests except for label-free quantitative proteomics experiment.

3. Results and discussion

3.1. AB4 exhibited antiviral efficacy in vitro

In a previous study, our group isolated AB4 from the roots of *P. chinesis*, and the chemical structure of AB4 was shown in Fig. 1A (He et al., 2019). First, the cytotoxicity of AB4 was evaluated using the Cell Counting Kit-8 (CCK-8) assay. The results showed that AB4

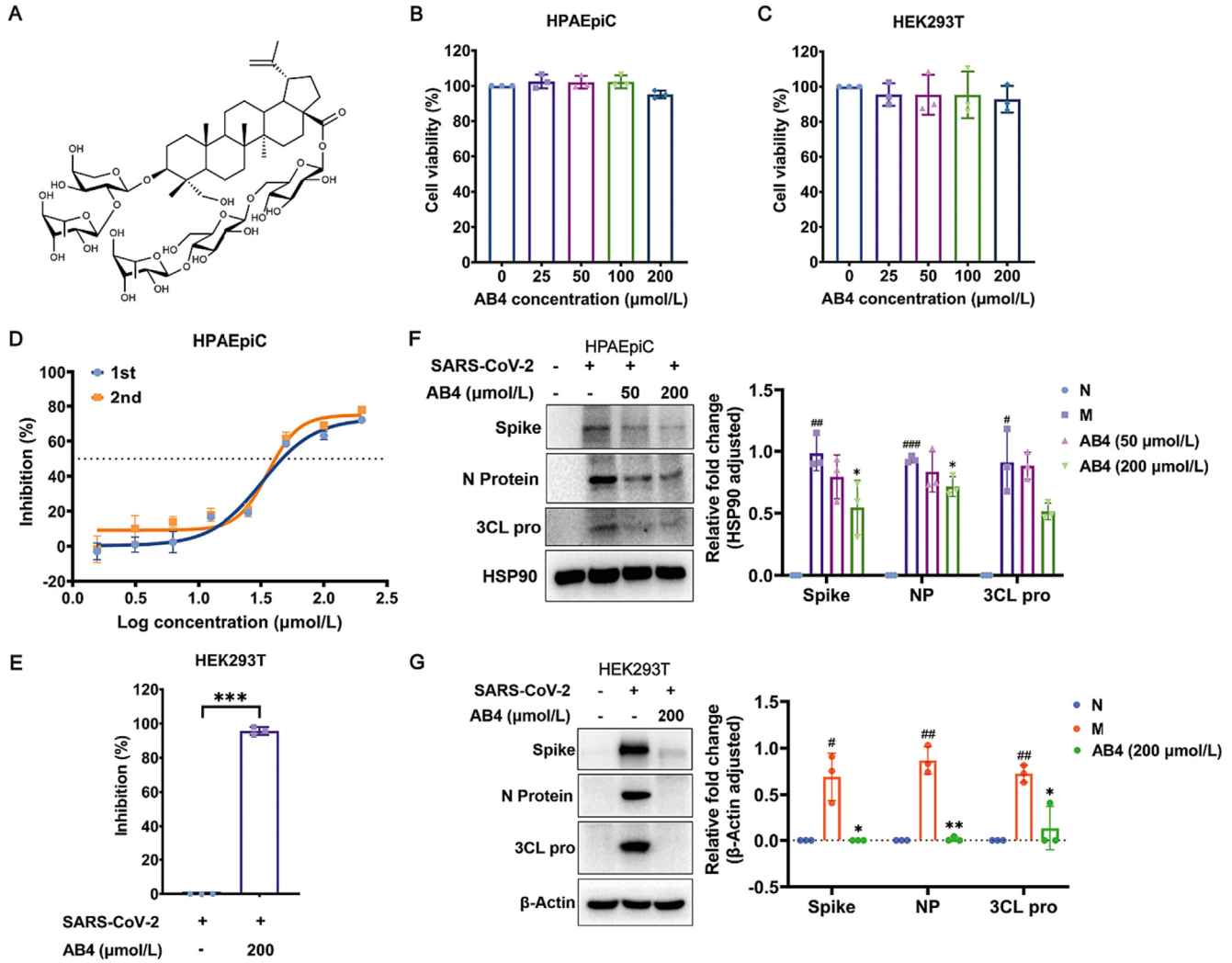

Fig. 1. Effects of AB4 on the replication of SARS-CoV-2 *in vitro*. (A) Chemical structures of AB4. Effect of AB4 on the cell viability of HPAEpiC (B) and HEK293T (C) cells using CCK-8 assay. HPAEpiC cells (D) or HEK293T cells (E) were treated with AB4 for 3 or 4 h before cells were infected with SARS-CoV-2 at an MOI of 0.1 or 1 for 1 h. Viral RNA copies (per ml) were quantified from cell culture supernatants by RT-qPCR. Viral protein expression in SARS-CoV-2-infected HPAEpiC cells and the statistics (F). Viral protein expression in SARS-CoV-2-infected HEK293T cells and the statistics (G). Data were expressed as mean ± SD, $n = 5$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* vehicle group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* SARS-CoV-2-infected group.

had no significant effect on the cell viability of HPAEpiC and HEK293T cells at the concentration of 200 μmol/L (Fig. 1B and C). Next, RT-qPCR experiment was used to evaluate the antiviral effects of AB4 on SARS-CoV-2 replication in SARS-CoV-2 infected HPAEpiC and HEK293T cells. The data showed that AB4 dose-dependently decreased the copy of SARS-CoV-2 with an IC_{50} value of (34.18 ± 2.59) μmol/L in HPAEpiC cells (Fig. 1D). At the same time, it also significantly inhibited the replication of SARS-COV-2 virus in HEK293T cells, especially the viral expression levels barely detectable in cells treated with 200 μmol/L AB4 (Fig. 1E). Consistently, the levels of intracellular viral proteins including SARS-CoV-2 nucleoprotein (NP), spike protein (S), and 3CL Pro were significantly inhibited by AB4 in a dose-dependent manner (Fig. 1F – G). Taken together, these results suggested that AB4 exhibited a strong antiviral effect on the replication of SARS-CoV-2 *in vitro*.

3.2. AB4 exhibited antiviral efficacy in vivo

Given the promising antiviral potency of AB4 *in vitro*, we further assessed its performance *in vivo* using an hACE2 transgenic mouse model. hACE2 transgenic mice were intranasally inoculated with SARS-CoV-2 (1×10^4 PFU virus per mouse) and were then treated with vehicle (control) or AB4 (2.5, 5 mg/kg, i.p.) for 4 d (Fig. 2A). As shown in Fig. 2B, AB4 significantly reduced viral load in the lung tissue of hACE2 mice infected with SARS-CoV-2 by intraperitoneal

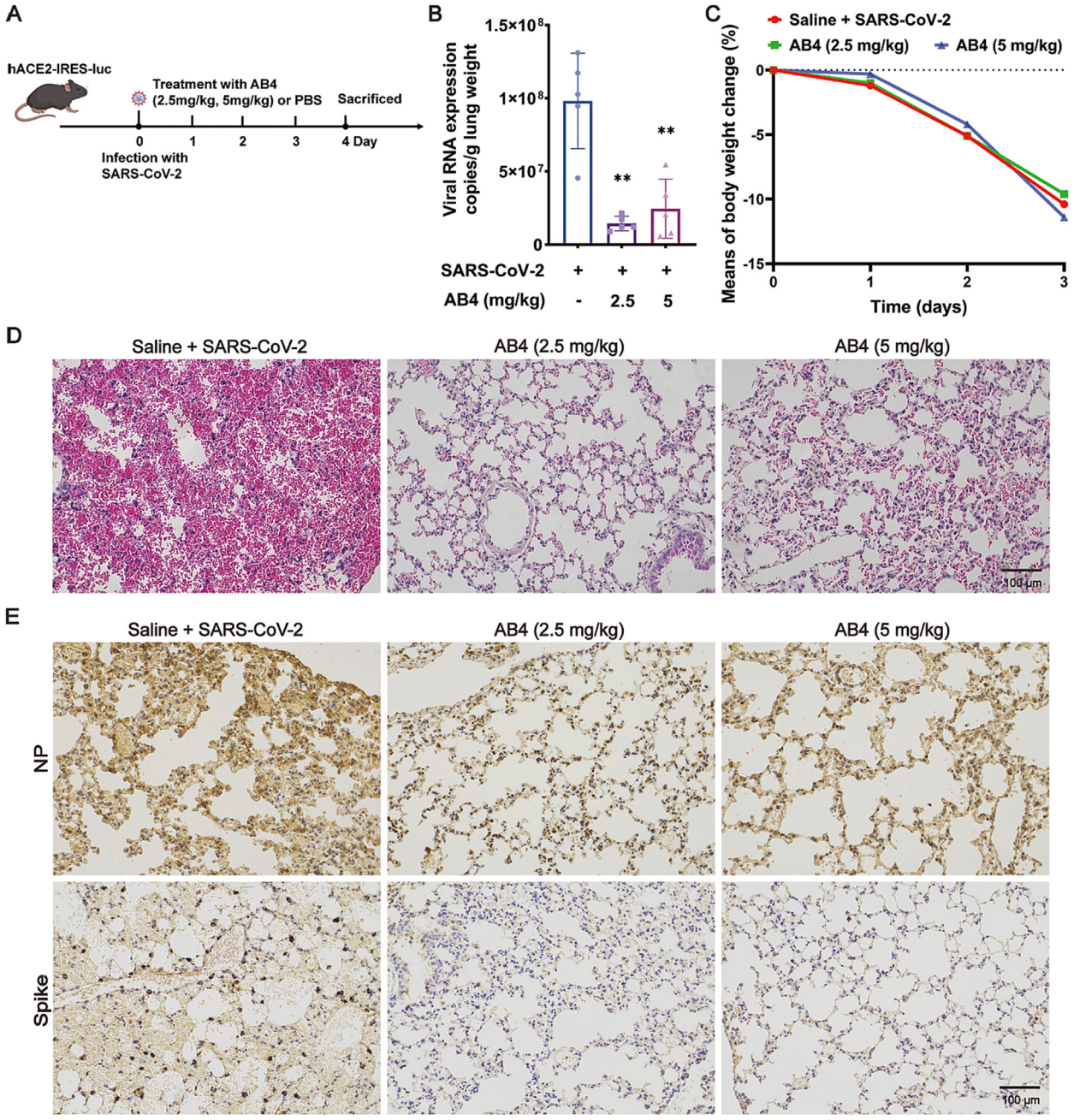

Fig. 2. Effects of AB4 on the replication of SARS-CoV-2 *in vivo*. (A) Timeline of SARS-CoV-2-infected mice in the *in vivo* experiment. (B) The relative SARS-CoV-2 viral RNA copy number in the lung tissue of mice was determined by RT-qPCR after AB4 treatment (2.5, 5 mg/kg). (C) Body weight changes of the mice. (D) H&E staining of lung tissues. Representative images are shown; scale bar: 100 μm. (E) IHC staining of lung tissues. Representative images are shown; scale bar: 100 μm. Data were expressed as mean ± SD, $n = 5$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* vehicle group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* SARS-CoV-2-infected group.

injection at doses of 2.5 mg/kg and 5.0 mg/kg. Meanwhile, during the four days, no abnormal behaviors or body weight loss were observed in the AB4 group or control group (Fig. 2C).

SARS-CoV-2 can cause lung pathological changes, such as inflammation and pulmonary hemorrhage. To further study the effect of AB4 against SARS-CoV-2 infection, histopathological analysis was performed using the lung tissues of mice infected with SARS-CoV-2. HE staining showed that the vehicle-treated mice presented moderate alveolar septal thickening and inflammatory cell infiltration, whereas AB4-treated animals exhibited slight alveolar septal thickening and mild inflammatory cell infiltration (Fig. 2D). Additionally, immunohistochemical (IHC) staining was also performed in lung tissues to measure the level of the key virus proteins. The results showed a significant reduction of N and S proteins in AB4 groups, compared with mock-treated lung tissues (Fig. 2E). These data indicated that AB4 could efficiently inhibit SARS-CoV-2 replication and ameliorate SARS-CoV-2-induced lung lesions *in vivo*.

3.3. AB4 exerts its antiviral effect by elevating interferon pathway

Innate immunity serves as the host's first line of defense against pathogen invasion, it actively increases IFN release and activates downstream signaling pathways of IFN that induce a diverse set of IFN-stimulated genes and protect host cells against the invading virus (Madden & Diamond, 2022). Therefore, we hypothesized that AB4 might play an anti-SARS-CoV-2 role through the type I IFN pathway. To do this, immunoblot analysis was used to measure the effect of AB4 on the level of IFNβ. The data showed that AB4 significantly increased the level of IFNβ at 24 h after HEK293T cells were infected by SARS-CoV-2.

As we know that antiviral innate immune responses can be triggered by multiple cellular receptors sensing viral components, such as retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). RLRs recruit the mitochondrial antiviral adaptor protein (MAVS) which also recruits multiple downstream signaling components to the mitochondria, leading to the activation of the inhibitor of κB kinase ε (IKKε) and TBK1, which in turn phosphorylate IRF3. pIRF3 forms a dimer and translocates to the nucleus, activating the transcription of type I IFN (IFN-I) genes. The secreted IFNβ then binds into the IFN receptors and activates the activator of transcription proteins (STAT1 and STAT2), finally leading to the trigger of the expression of hundreds of ISGs with antiviral functions, for instance, ISG15. ISG15 is one of the most strongly and rapidly induced, and recent work has shown that it can directly inhibit viral replication and modulate host immunity. However, the key feature of SARS-CoV-2 is dysregulated or delayed IFNs-response. In the present study, AB4 significantly promoted TBK1, IRF3 phosphorylation, and MAVS, which are the three key steps in the IFNβ signalling pathway, to increase IFN levels (Fig. 3A and B).

Upon SARS-CoV-2 infection, PLpro acts as a protease to cleave ISG15 from IRF3 and attenuates IFN responses. We wonder if AB4 could partially moderate ISG15 and lead to the inhibited proliferation of SARS-CoV-2. Indeed, our study showed that AB4 could promote ISGylation after cells were infected with SARS-CoV-2 (Fig. 3C).

Unlike normal mammalian cells, Vero cells cannot secrete IFNβ when they were infected with viruses. Then, Vero cells were used to evaluate the antiviral activity of AB4 without the influence of the interferon signaling pathway. Unexpectedly, AB4 has weaker antiviral activity in Vero E6 cells (Fig. 3D), compared with in HPAEpiC and HEK293T cells. In addition, an IHC staining was performed

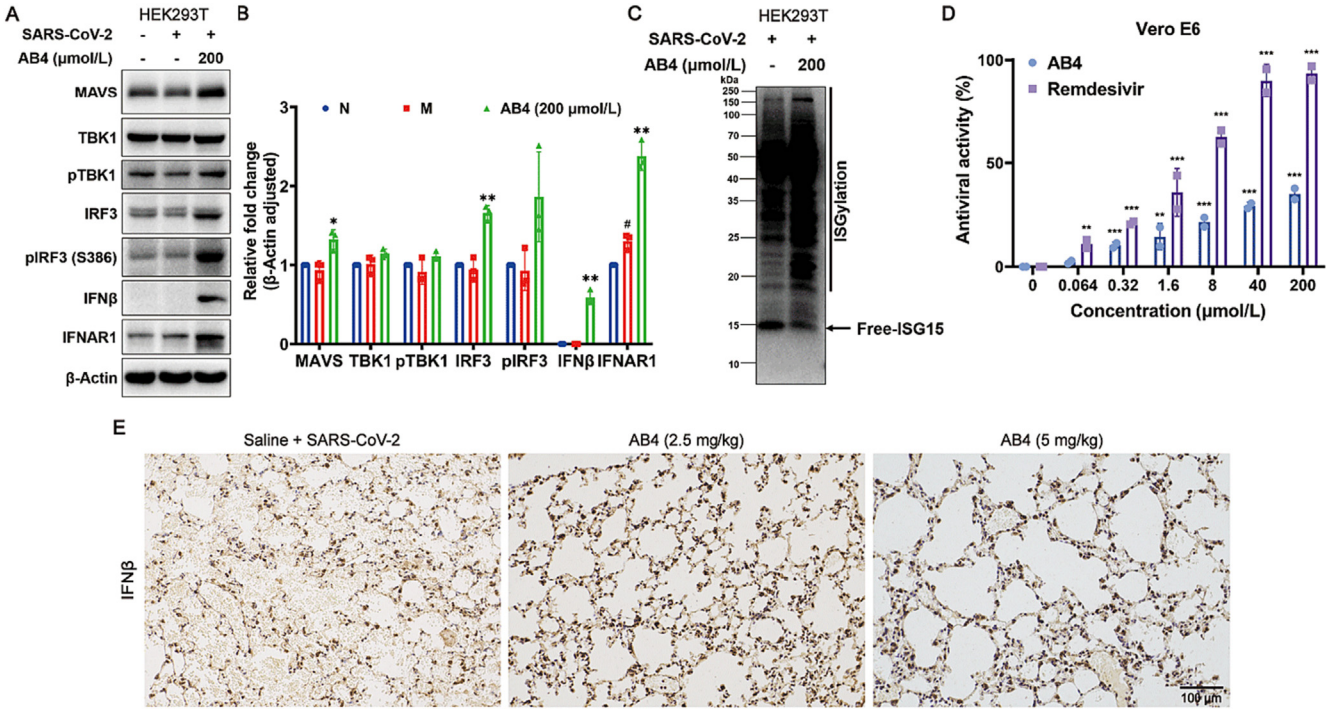

Fig. 3. AB4 inhibited viral replication through IFN signaling pathway and modulated protein expression in cells infected with SARS-CoV-2. HEK293T cell lysates were analyzed by Western blotting for IFNβ signal pathway (A – B) and ISG15 (C). (D) Vero E6 cells were pretreated with AB4 (0–200 μmol/L) for 4 h before viral infection. Cells were infected with SARS-CoV-2 at an MOI of 0.1. At 3 dpi, the cytopathic effect caused by SARS-CoV-2 infection was quantitatively analyzed using CCK-8 according to the manufacturer's protocol. n = 2 biological replicates. (E) IHC staining of lung tissues for IFNβ. Representative images are shown; Scale bar: 100 μm. Data were expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* vehicle group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* SARS-CoV-2-infected group.

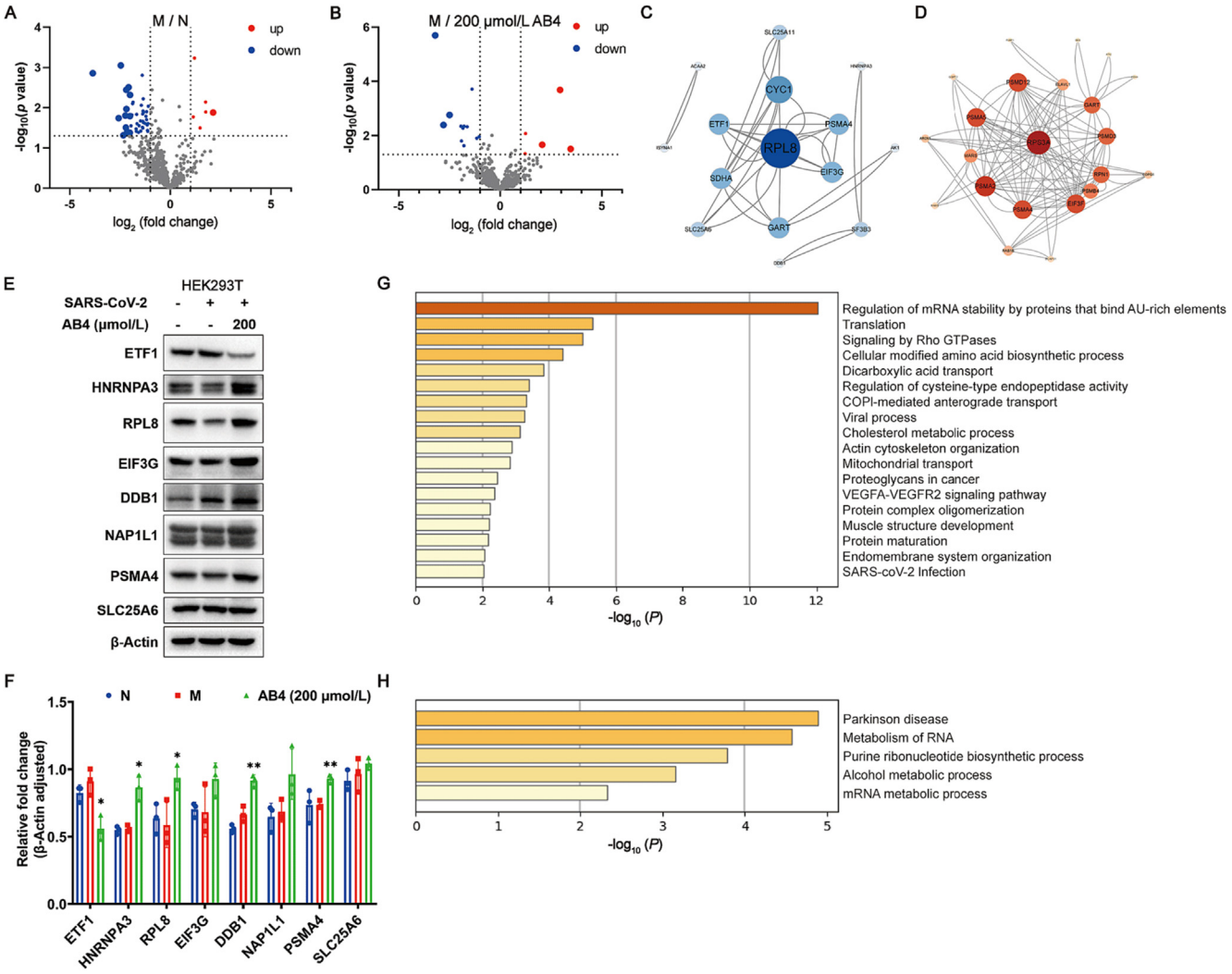

Fig. 4. Volcano plots of proteins for SARS-CoV-2 group versus uninfected group (A), and SARS-CoV-2 group versus AB4 group (B). Upregulated proteins are represented in red while proteins downregulated are represented in blue. FDR < 0.01, fold change > 2 or < 0.5 and $P < 0.05$. (C, D) Cytoscape network of differentially abundant SARS-CoV-2 stimulated proteins and AB4 regulated proteins. Proteins are represented as circles. The size of the circle is proportional to the fold change. (E, F) Western blotting of HEK293T cell lysates with the indicated antibodies. (G, H) Pathway analysis was performed using Metascape for differentially expressed proteins in SARS-CoV-2 group *vs* normal group and SARS-CoV-2 group *vs* AB4 group.

in lung tissues and showed that AB4 increased the level of IFNβ (Fig. 3E). Altogether, the data suggested that AB4 exerted mainly anti-SARS-CoV-2 effects through IFN signaling pathway.

3.4. Target profiling of AB4 by label-free quantitative proteomics

To further investigate the mechanisms of the therapeutic effect of AB4 on SARS-CoV-2 infected cells, we performed a label-free quantitative proteomics to identify the differentially expressed proteins in the HPAEpiC cells among an uninfected group, SARS-CoV-2 group, and AB4 (200 μmol/L) group. The results showed that 656 proteins were identified in the uninfected group and the SARS-CoV-2 group. Among them, six proteins were upregulated, and 44 proteins were downregulated (Fig. 4A). In addition, 568 proteins were identified in the SARS-CoV-2 group and the AB4 group, five proteins were upregulated, and 12 proteins were downregulated (Fig. 4B). These 17 proteins were potential targets for AB4. The protein–protein interaction (PPI) network for these 17 proteins was constructed via the Cytoscape. The PPI network revealed potential connections between the targets. The color of a node reflected the degree of importance. The larger the degree, the more important the node was in the network. The results suggest that RPL8 may be a key target of AB4 (Fig. 4C and D). Meanwhile, we verified the proteomic results by using Western blotting (Fig. 4E and F), which confirmed that ETF1, HNRNPA3, RPL8, DDB1, and PSMA4 had a significant difference in the AB4 group compared to the SARS-CoV-2 group. Pathway analysis was performed using Metascape (https://metascape.org) (Fig. 4G and H). Collectively, these data showed that the differentially regulated proteins were mainly clustered in metabolism of RNA, mRNA metabolic process,Parkinson's disease, purine ribonucleotide biosynthetic process, and alcohol metabolic process. Our results revealed that AB4 exerted anti-SARS-CoV-2 effects partly via regulating RNA metabolism.

4. Conclusion

We have found for the first time that AB4 inhibits the replication of the SARS virus *in vitro* and *in vivo*. In SARS-CoV-2 infected hACE2-IRES-luc transgenic mouse, AB4 alleviated alveolar septal thickening and inflammatory cell infiltration in lung tissues. We further disclosed that the antiviral mechanisms of AB4 was associated with the enhanced interferon (IFN)-β response via the activation of retinoic acid-inducible gene I (RIG-1) like receptor (RLP) pathways and moderated the RNA metabolism. In addition, pro-

teomics was performed to find eight potential target protein of AB4. Taken together, AB4 is likely to become a new therapeutic drug for SARS-CoV-2. Although the mechanisms of protection by AB4 *in vivo* require further study, the current data suggest that AB4 may be an effective antiviral candidate for SARS-CoV-2.

CRediT authorship contribution statement

Mingyue Xiao: Investigation, Writing – original draft, Visualization. **Ronghua Luo:** Methodology, Investigation. **Qinghua Liang:** Methodology. **Honglv Jiang:** Formal analysis, Software. **Yanli Liu:** Conceptualization, Writing – original draft, Supervision. **Guoqiang Xu:** Resources. **Hongwei Gao:** Supervision, Resources. **Yongtang Zheng:** Validation. **Qiongming Xu:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition. **Shilin Yang:** Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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