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In vitro and in vivo effects of 3-indoleacetonitrile—A potential new broad-spectrum therapeutic agent for SARS-CoV-2 infection

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ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak has resulted in significant global morbidity, mortality, and societal disruption. Currently, effective antiviral drugs for the treatment of SARS-CoV-2 infection are limited. Therefore, safe and effective antiviral drugs to combat COVID-19 are urgently required. In previous studies, we showed that 3-indoleacetonitrile, a plant growth hormone produced by cruciferous (Brassica) vegetables, is effective in treating influenza A virus infection. However, the molecular mechanisms underlying these effects remain unclear. Herein, we demonstrated that 3-indoleacetonitrile exhibits broad-spectrum antiviral activity and is effective against HSV-1 and VSV infections in vitro. This phenomenon prompted us to study its role in the anti-SARS-CoV-2 process. Interestingly, 3-indoleacetonitrile exhibited antiviral activity against SARS-CoV-2 in vivo. Importantly, tail vein injection of 3-indoleacetonitrile resulted in good antiviral activity in mouse models infected with WBP-1 (a mouse adaptation of the SARS-CoV-2 strain). Mechanistically, 3-indoleacetonitrile promoted the host interferon signalling pathway response and inhibited autophagic flux. Furthermore, we demonstrated that 3-indoleacetonitrile induced an increase in mitochondrial antiviral-signalling (MAVS) protein levels, which might be attributed to its inhibition of the interaction between MAVS and the selective autophagy receptor SQSTM1. Overall, our results demonstrate that 3-indoleacetonitrile is potently active against SARS-CoV-2 in vitro and in vivo, which may provide a foundation for further clinical testing for the treatment of COVID-19. In addition, considering its broad-spectrum antiviral effect, it should be explored whether it also has an effect on other viruses that threaten human health.

1. Introduction

Outbreaks of respiratory diseases such as influenza, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS-), and the novel coronavirus (SARS-CoV-2) have taken a heavy toll on human populations worldwide and have seriously diminished local and national resources (Sands et al., 2016). Vaccines and antiviral drugs are key to controlling and eradicating viral pathogens (Zhu et al., 2019). Owing to the error-prone nature of viral replication, RNA viruses accumulate mutations over time, resulting in sequence diversity. This could limit the effectiveness of vaccines and drugs targeting the virus. For instance, influenza vaccines must be reformulated each year owing to antigenic shifts and drift (Jin and Chen, 2014). Although there is no evidence regarding antigenic drift for SARS-CoV-2, with extended human-to-human transmission, SARS-CoV-2 may also acquire mutations with fitness advantages and immunological resistance (Korber et al., 2020). Rapid development of effective broad-spectrum antiviral therapies to prevent and inhibit viral infections is of great societal importance. Antivirals that target host cell processes have great potential to exhibit activity against a range of viruses.

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Several bioactive compounds derived from various pharmacological medicinal plants have been extensively studied for their potential as viral inhibitors. For example, a naturally occurring alkaloid extracted from the plant *Stephania cepharantha* Hayata has received particular attention in recent years of research in COVID-19 therapy (Ohashi et al., 2021).

Our previous studies have revealed that 3-indoleacetonitrile, a small molecule naturally produced by plant sources, is effective in treating influenza A virus infection *in vitro* and *in vivo* (Zhao et al., 2021). However, the molecular mechanism underlying the inhibitory effect against viral proliferation remains unclear. Herein, we found that 3-indoleacetonitrile significantly inhibited the proliferation of vesicular stomatitis virus (VSV, a negative-strand RNA virus) and herpes simplex virus type 1 (HSV-1, a double-stranded DNA virus) in 293T and Vero E6 cells, indicating that 3-indoleacetonitrile exhibits broad-spectrum antiviral efficacy. To date (July 12, 2022), over 554 million confirmed cases and 6.3 million deaths have been reported worldwide (World Health Organisation data). Currently, effective targeted treatment options remain limited and highly restricted. Given the urgent need for therapeutics to treat SARS-CoV-2 infection, 3-indoleacetonitrile has shown broad-spectrum antiviral efficacy. Therefore, we investigated the antiviral efficacy of 3-indoleacetonitrile against SARS-CoV-2 and determined the molecular mechanism underlying its broad-spectrum antiviral efficacy.

2. Materials and methods

2.1. Cells and virus

The human hepatoma cell line (HuH7.0), Vero E6 cells, Vero cells, human epithelial cell line (Caco-2), and human cervical cancer cell line (HeLa) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan, Utah, USA), and 293T cells were maintained in Roswell Park Memorial Institute (RPMI) medium (HyClone, Logan, Utah, USA). Medium was supplemented with 10% foetal bovine serum (FBS) (PAN Biotech, Edenbach, Bagoria, Germany) at 37 °C in a 5% CO₂ humidified atmosphere.

SARS-CoV-2 virus stocks were prepared in Vero cells, and viral titres were determined using plaque assays. Strain WBP-1 (EPI ISL 1615558) was established by continuously passaging the wild virus in the lungs of aged mice. All infection experiments were performed in a biosafety level-3 (BSL-3) laboratory. The recombinant vesicular stomatitis virus encoding enhanced green fluorescence protein (VSV-eGFP) was a gift from the Harbin Veterinary Research Institute, and eGFP expression levels directly reflected the degree of VSV replication. Recombinant herpes simplex virus type 1 encoding enhanced green fluorescence protein (HSV-1 eGFP) was a gift from the College of Life Sciences, Wuhan University.

2.2. Virus infection and antiviral evaluation of 3-indoleacetonitrile *in vitro*

SARS-CoV-2 (VSV, HSV-1 or SeV) was diluted to a final virus titer with serum free medium. The diluted virus was then added to cells approximately 90% confluent. After 60 min incubation at 37 °C, the cells were gently washed twice with serum free medium to remove unbound virus and maintenance medium (1% serum medium, 3-indoleacetonitrile or DMSO were added to this medium) was added.

2.3. Cell counting Kit-8 assay

Cell viability assay was performed according to the manufacturer’s instructions from TransDetect CCK as previously described (Yuan et al., 2021).

2.4. SDS-PAGE and western blotting

For the SDS-PAGE assay, protein samples were prepared using cell lysis buffer for western blotting and immunoprecipitation (IP) (Beyotime, Shanghai, China), containing an EDTA-free protease inhibitor cocktail (Bimake, Houston, Texas, USA). After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (GE Healthcare, Boston, MA, USA). After blocking with 2% bovine serum albumin (BSA) (Biofro, Germany) in PBS, the membranes were incubated with corresponding primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies.

2.5. Reporter assays

Herein, 293T cells cultured in 12 well plates were transfected with the luciferase reporter plasmids and Renilla control plasmids pRL-TK. About 24 h after transfection, the cells were treated with 3-Indoleacetonitrile or DMSO. Cells were then harvested, and cell lysates were used to measure luciferase levels with a dual luciferase reporter assay system (Promega, Madison, Wisconsin, USA). Luciferase reporter plasmid containing IFN-β-promoter, NFκB-promoter, ISRE promoter, and Renilla control plasmids pRL-TK were given by Dr. Ping Qian (Huazhong Agricultural University).

2.6. Immunofluorescence analysis

For immunofluorescence analysis, the cells to be tested were fixed with 4% paraformaldehyde (PFA) at 25 °C for 30 min and treated with 0.1% (v/v) Triton X-100 for 10 min. The cells were incubated in 2% (v/ v) BSA for 1 h at 25 °C. The cells were then incubated with the designated primary antibody for 2 h and then with the appropriate Alexa Fluor-conjugated secondary antibody for 1 h. Finally, DAPI staining was used to visualise the DNA according to the purpose of the experiment. The cells were observed using a LEISS confocal microscope under a 60 × oil objective.

2.7. Virus plaque assay

The supernatants were obtained 24 h after infection for the virus plaque assay. Briefly, the sample to be tested was diluted in basel DMEM and confluent Vero cells in 12-well plates were inoculated with the samples and incubated at 37 °C for 1 h. The cells were washed twice with PBS to remove unbound viruses. Subsequently, the cells were covered with 1 mL of 4% sodium carboxymethyl cellulose (containing 1% FBS) per well. After culturing for 72 h, when the cells showed lesions or plaques, each well was filled with 10% neutral formaldehyde and fixed for more than 4 h. Finally, plaques were stained with 0.1% crystal violet and counted.

2.8. Antibodies and reagents

Mouse anti-SARS-CoV-2 nucleocapsid antibodies were purchased from Sino Biological (Beijing, China) (cat. no. 40143-MM05). A rabbit anti-influenza A virus nucleoprotein (NP) antibody (cat. no. GTX125989) and anti-influenza A virus M1 (matrix protein) (cat. no. GTX125928) were purchased from GeneTex (SAN Antonio, Texas, USA). The rabbit anti-GAPDH antibody (cat. no. GTX100118) was purchased from GeneTex. Horseradish peroxidase (HRP)-conjugated anti-mouse antibody (cat. no. AS003) and anti-rabbit (cat. no. AS014) secondary antibodies were purchased from Abclonal Technology (Wuhan, China). The CoraLite 488-conjugated goat anti-mouse antibody (cat. no. SA0013-3) and CoraLite 594-Conjugated goat anti-rabbit (cat. no. CL594-10594) were purchased from Proteintechn (Chicago, Illinois, USA). Rabbit anti-LC3B antibody (cat. no. 38685S) were purchased from Cell Signalling Technology (Boston, Massachusetts, USA). Rabbit anti-p62 antibody (cat. no. PM045) polyclonal antibodies and mouse anti-
tubulin antibodies (cat. no. M175-3) monoclonal antibody were purchased from MBL (Tokyo, Japan). Rabbit anti-IRF3 (cat. no. A11373) and anti-Phospho-IRF3-S396 (cat. no. AP0623) antibodies were purchased from ABclonal Technology. Rabbit anti-NF-κB p65 (cat. no. 10745-1-AP) antibody were purchased from Proteintech. Rabbit anti-IRF3 (cat. no. A11373) monoclonal antibody were purchased from Cell Signalling Technology.

2.9. Flow cytometric analyses

293T/Vero E6 cells were infected with VSV/HSV-1 at a MOI of 0.1 for 24 h. For flow cytometric analyses, the cells were digested by Trypsin-EDTA (0.5%), and fixed with 4% paraformaldehyde (PFA) at room temperature for 30 min. All flow cytometric analyses were performed using a Beckman Coulter EPICS XL flow cytometer (Beckman Coulter).

2.10. RNA isolation and quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, California, USA), in accordance with the manufacturer’s instructions. Genomic DNA was removed using DNase I, and 2 μg of RNA was reverse-transcribed using avian myeloblastosis virus reverse transcriptase (TaKaRa Biotechnology, DaLian, China) and an oligo (dT)18 primer. GAPDH was used as a reference to normalise the amount of mRNA in each sample. The sequences of all the primers used for qRT-PCR are available from the corresponding author upon request.

2.11. Animal studies and ethics statement

Female 6-7-week-old BALB/c mice were used in the experiments. The mice were purchased from the Center for Animal Disease Control, Hubei Province, China. Animal experiments were approved by the Research Ethics Committee of Huazhong Agricultural University, Hubei, China (approval no. HZAUMO-2020-0007). All animal experiments were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Research Ethics Committee, Huazhong Agricultural University, Hubei, China.

To determine the effect of 3-indoleacetonitrile on mice’s viral infection mortality, mice were treated with 3-indoleacetonitrile (20 mg/kg) or PBS (Contains 0.5% DMSO) via tail-vein injection. After that, the mice were administered with 20 mg/kg 3-indoleacetonitrile or DMSO on day 0/2/4 post-infection. The body weights and survival of the mice in each group were monitored daily for 10 days post-infection (dpi). Another 4 groups of mice were independently set for sample preparation, and three mice per group were euthanised at 5 dpi. The right lungs of the mice were removed to determine the virus titers, and the left lungs were fixed in formalin for histopathological analysis.

2.12. Statistical analysis

Differences between the experimental and control groups were determined using Student’s t-test (where two groups of data were compared) or two-way ANOVA analysis (where more than two groups of data were compared). P values less than 0.05 were considered statistically significant. For animal survival analysis, the Kaplan–Meier method was used to generate graphs, and survival curves were analysed using log-rank analysis.

3. Results

3.1. 3-Indoleacetonitrile is effective in the treatment of VSV/HSV-1 virus infection in vitro

To investigate the antiviral effect of 3-indoleacetonitrile against VSV (a negative-strand RNA virus) and herpes simplex virus type 1 (HSV-1, a double-stranded DNA virus), cell viability after 3-indoleacetonitrile treatment was determined using the CCK-8 assay in both 293T and Vero E6 cells. 3-indoleacetonitrile showed unapparent cytotoxicity in 293T cells at concentrations of up to 640 μM, while at concentrations up to 320 μM, there was no obvious cytotoxicity in Vero E6 cells (Fig. 1A and B). 293T/Vero E6 cells were infected with the VSV/HSV-1 virus and incubated with 3-indoleacetonitrile at 640 or 320 μM for 24 h. Recombinant VSV and HSV-1 encoding enhanced green fluorescence protein and eGFP expression levels directly reflected the degree of viral replication. As shown in Fig. 1C-F, the densities of GFP fluorescence of VSV or HSV-1 were significantly decreased upon treatment with 3-indoleacetonitrile in 293T and Vero E6 cells under microscopy. We further measured the population of GFP-positive cells using flow cytometry, which revealed inhibited viral proliferation in 3-indoleacetonitrile-treated cells, in line with the microscopic observations. These results established the in vitro broad-spectrum antiviral function of 3-indoleacetonitrile.

To explore whether 3-indoleacetonitrile plays an antiviral effect in vivo by affected the amount of incoming viruses, we added 3-indoleacetonitrile or DMSO at the same time of virus infection, and detected the virus load by qPCR immediately after 1 h of infection. The results show that 3-indoleacetonitrile did not significantly affect the amount of incoming VSV and HSV-1 viruses than the DMSO groups (Fig. 1G and H).

3.2. 3-Indoleacetonitrile hampers SARS-CoV-2 replication in vitro

The COVID-19 pandemic caused by SARS-CoV-2 remains a current focus of research. Based on the broad-spectrum antiviral function of 3-indoleacetonitrile, we investigated the anti-SARS-CoV-2 effect of 3-indoleacetonitrile in vitro. Initially, the cytotoxicity of 3-indoleacetonitrile in Caco-2 and HuH7.0 cells was measured using CCK-8 assays. The cells were then infected with SARS-CoV-2 (WBP-1 strain) at an MOI of 1 in the presence of varying concentrations of 3-indoleacetonitrile. At 24 h after infection, the viral yield in the cell supernatant was quantified using the virus plaque assay (Fig. 2A and B). These results indicate that the EC50 of 3-indoleacetonitrile against SARS-CoV-2 is not less than 38.79 μM. The effect of 3-indoleacetonitrile on virus replication was confirmed by measuring cellular viral N protein levels (Fig. 2C and D). After immunofluorescence microscopy to further confirm virus nucleoprotein (NP) expression, the densities of FITC fluorescence (stained N protein) were observed to be obviously decreased upon treatment with 3-indoleacetonitrile (Fig. 2E). In addition, to explore whether 3-indoleacetonitrile plays an antiviral effect in vitro by affected the amount of incoming viruses, we added 3-indoleacetonitrile or DMSO at the same time of virus infection, and detected the virus load by qPCR immediately after 1 h of SARS-CoV-2 infection. The results showed that 3-indoleacetonitrile did not significantly affect the amount of incoming SARS-CoV-2 than the DMSO control in Caco-2 and HuH7 cells (Fig. 2F and G).

3.3. 3-Indoleacetonitrile against SARS-CoV-2 in vivo

To further evaluate the potential anti-SARS-CoV-2 effect of 3-indoleacetonitrile in vivo, mice were injected into the tail vein with 3-indoleacetonitrile after immediate intranasal infection with the WBP-1 virus (a mouse adaptation of SARS-CoV-2 strain) (Huang et al., 2021) and further treated with 3-indoleacetonitrile or PBS once every two days for four consecutive days (Fig. 3A), the dose of 3-indoleacetonitrile (20 mg/kg) used was as determined in our previous study (Zhao et al., 2021). In the next 10 consecutive days, we observed that mice in the SARS-CoV-2 infection+3-indoleacetonitrile treatment group exhibited less weight loss than those in the SARS-CoV-2 infection + PBS treatment group (Fig. 3B). On the seventh day after infection, the weight loss of mice in the SARS-CoV-2 infection+3-indoleacetonitrile treatment group began to increase. At this point in time, all mice in the SARS-CoV-2 infection + PBS treatment group died or were euthanised due to loss of more than 30% of their body weight. However, in the SARS-CoV-2
Fig. 1. Antiviral activity of 3-indoleacetonitrile against VSV/HSV-1 virus. Cytotoxic effect of 3-indoleacetonitrile on 293T cells (A) and Vero E6 cells (B). Data are presented as mean ± SD. The experiments were performed in triplicate. (C–D) 293T cells (C) or Vero E6 cells (D) were infected with the VSV-GFP virus at an MOI of 0.1, followed by treatment with 3-indoleacetonitrile at 640 μM concentrations for 24 h. (E–F) 293T cells (E) or Vero E6 cells (F) were infected with the HSV-1-GFP virus at an MOI of 0.1, followed by treatment with 3-indoleacetonitrile at 640 μM concentrations for 24 h. (G–H) 3-indoleacetonitrile (640 μM) or DMSO was added at the same time of VSV (G) or HSV-1 (H) infection of 293T cells, and the viral load in the cells was detected by qPCR after infection. Data are expressed as mean ± SD. Statistical analyses used Student’s t-test. P < 0.05 was considered statistically significant.
Fig. 2. The antiviral activity of 3-indoleacetonitrile against SARS-CoV-2 in vitro. (A–B) Caco-2 cells (A) and Huh7.0 cells (B) were infected with SARS-CoV-2 at an MOI of 1 in the treatment of different doses of 3-indoleacetonitrile for 24 h. The viral yield in the cell supernatant was quantified using virus plaque assay. Cytotoxicity of 3-indoleacetonitrile against Caco-2 and Huh7.0 cells was measured using CCK-8 assays. The left and right Y-axes of the graphs represent the mean % cell viability and % inhibition of virus yield, respectively. (C) Caco-2 cells were infected with SARS-CoV-2 at an MOI of 1 in the treatment of different doses of 3-indoleacetonitrile (0 μM, 320 μM, 640 μM, 1280 μM) for 24 h. The viral yield in the cell lysates was then quantified using western blotting. (D) Huh7.0 cells were infected with SARS-CoV-2 at an MOI of 1 in the treatment of different doses of 3-indoleacetonitrile (0 μM, 80 μM, 160 μM, 320 μM) for 24 h. The viral yield in the cell lysates was then quantified using western blotting. (E) Immunofluorescence microscopy of virus infection upon treatment with 3-indoleacetonitrile (640 μM). At 24 h post infection (p.i.), the infected Caco-2 cells were fixed, and then probed with rabbit sera against the NP of a SARS-CoV-2 as the primary antibody and Alexa 488-labelled goat anti-rabbit IgG as the secondary antibody, respectively. Scale bars, 200 μm. (F–G) 3-indoleacetonitrile or DMSO was added at the same time of SARS-CoV-2 infection of Caco-2 (F) or Huh7.0 cells (G), and the viral load in the cells was detected by qPCR 1 h after infection. Data are expressed as mean ± SD. Statistical analyses used Student’s t-test. P < 0.05 was considered statistically significant.
infection + 3-indoleacetonitrile treatment group, the mice died slightly later, and with a final survival rate of 40% (Fig. 3C). Gross pathologic examination of the lungs showed that the lungs of the uninfected group (PBS treatment control and 3-indoleacetonitrile treatment control) sacrificed at 5 dpi appeared whitish-pink. All infected mice had comparable gross lesions with focal to multifocal dark red discoloration in the lung lobes, while the SARS-CoV-2 infection + 3-indoleacetonitrile treated group had fewer lung lesions than those treated with SARS-CoV-2 infection + PBS treated group (Fig. 3D). Haematoxylin and eosin (H&E) staining of the lung tissue revealed that inflammatory lung
imply that 3-indoleacetonitrile activates the IFN signalling pathways.

and a Renilla luciferase plasmid (pRL-TK) and subjected to dual lucif

induced by MAVS, TBK1, IKK (IRF3) and NF-

regulated by two transcription factors, interferon response factor 3

signalling pathways

context of viral infection (VSV and HSV-1) (Fig. 4 D and E). These data

results of nucleocytoplasmic separation and IFA experiments showed that

immunity. To verify that 3-indoleacetonitrile could induce IFN produc-

duction, we conducted a series of experiments, wherein 293T cells were co-

plasmids encoding the IFN-β promoter (pGL4–IFN-β) and a Renilla luciferase plasmid (pRL-TK) and subjected to dual luciferase reporter (DLR) assays to detect promoter activity (Fig. 4A and B), and qPCR analysis of 3-indoleacetonitrile-induced type I IFN-β and IFN-κB independent genes (Fig. 4C) included ISG15, IFI15, IFIT3, CXCL10, CXCL11, RNSTES, and VIREIBN. Furthermore, our results support a role for 3-indoleacetonitrile in regulating the quality of IFN-β mRNA in the context of viral infection (YSV and HSV-1) (Fig. 4D and E). These data imply that 3-indoleacetonitrile activates the IFN signalling pathways.

3.4. 3-Indoleacetonitrile positively regulates type I IFN production

Type I IFN is a known pathway that plays a critical role in antiviral immunity. To verify that 3-indoleacetonitrile could induce IFN production, we conducted a series of experiments, wherein 293T cells were co-transfected with plasmids encoding the IFN-β promoter (pGL4–IFN-β) and a Renilla luciferase plasmid (pRL-TK) and subjected to dual luciferase reporter (DLR) assays to detect promoter activity (Fig. 4A and B), and qPCR analysis of 3-indoleacetonitrile-induced type I IFN-β and IFN-κB independent genes (Fig. 4C) included ISG15, IFI15, IFIT3, CXCL10, CXCL11, RNSTES, and VIREIBN. Furthermore, our results support a role for 3-indoleacetonitrile in regulating the quality of IFN-β mRNA in the context of viral infection (YSV and HSV-1) (Fig. 4D and E). These data imply that 3-indoleacetonitrile activates the IFN signalling pathways.

3.5. 3-Indoleacetonitrile promotes activation of IRF3 and NF-κB signalling pathways

It is well established that the expression of type I IFN genes is mainly regulated by two transcription factors, interferon response factor 3 (IRF3) and NF-κB (Liu et al., 2017). To investigate the effect of 3-indoleacetonitrile induced activation of IRF3 and NF-κB, interferon-stimulated response element (ISRE) and NF-κB luciferase reporter constructs were transfected into 293T cells. We found that ISRE- and NF-κB-responsive luciferase activity induced by 3-indoleacetonitrile was dramatically higher than that of the DMSO control (Fig. 5A–D), indicating that 3-indoleacetonitrile is involved in both IRF3- and NF-κB-mediated type I IFN expression.

This was further confirmed through qPCR analysis of NF-κB mRNA levels in 293T and Caco-2 cells (Fig. 5E and F). Consistent with this observation, the phosphorylation of IRF3 and p65 was distinctly activated in 3-indoleacetonitrile-treated cells (Fig. 5G). Moreover, the results of nucleocytoplasmic separation and IFA experiments showed that IRF3 and p65 were transferred from the cytoplasm to the nucleus under the action of 3-indoleacetonitrile (Fig. 5H–J). These data indicate that 3-indoleacetonitrile promotes the activation of the IRF3 and NF-κB signalling pathways.

3.6. 3-Indoleacetonitrile treatment promotes mitochondrial antiviral-signalling (MAVS) accumulation and interfered with the completion of autophagy flux

Studies have shown that 293T cells do not express endogenous cGAS or STING (Stempel et al., 2019). Therefore, we excluded cGAS-STING and targeted the RIG-I-MAVS signalling pathway for further exploration. To better understand the role of 3-indoleacetonitrile in the RIG-I-MAVS pathway, we performed a luciferase reporter assay and found that 3-indoleacetonitrile increased the luciferase reporter activity induced by MAVS, TBK1, IKKc, and IRF3-5D (a constitutively active mutant of IRF3) but not RIG-I-N (the constitutively active N-terminal of RIG-I with two CARD domains) (Fig. 6A). This indicates that the stimulatory effect of 3-indoleacetonitrile on IFN-β activation occurs mainly through MAVS. Ubiquitin modification of the adaptor molecule MAVS is critical for the downstream signalling of IFN and IRF3 pathways (Seth et al., 2005). Next, we assessed the effects of 3-indoleacetonitrile treatment on MAVS ubiquitination and found that it did not signifi-
cantly change MAVS ubiquitination (Fig. 6B). However, we found that treatment with 3-indoleacetonitrile increased MAVS protein levels, but not mRNA levels, in A549 and 293T cells (Fig. 6C, D, and 6E). It has been suggested that 3-indoleacetonitrile may increase the downstream interferon levels by increasing MAVS protein levels. Therefore, we speculate that the increase in MAVS protein levels was due to repressed protein degradation during 3-indoleacetonitrile incubation. Interest-

ingly, in addition to the increase of MAVS, A549 (Fig. 6C) and 293T (Fig. 6D) cells treated with 3-indoleacetonitrile also increased the levels of selective autophagic receptor SQSTM1. To test whether 3-indoleaceto-

nitrile was associated with autophagy, GFP-LC3 transfected Vero E6 cells were treated with 3-indoleacetonitrile. As a positive control, chloroquine (CQ) treatment significantly promoted the formation of GFP-LC3 puncta, a marker of autophagosomes, due to its inhibitory role in the fusion of autophagosomes and lysosomes. Compared to untreated cells, 3-indoleacetonitrile treated Vero E6 cells showed a strong increase in GFP-LC3 puncta (Fig. 6F). We then detected LC3 and SQSTM1 protein levels after treatment with different concentrations of 3-indoleaceto-

The results showed that 3-indoleacetonitrile induced a dose-dependent increase in LC3-II and SQSTM1 (Fig. 6F). Further, we attempted to determine the completion of autophagy flux using a tandem reporter construct encoding LC3 fused to the RFP and EGFP genes, RFP-EGFP-LC3. The GFP signal is attenuated in an acidic pH environment, while RFP can be visualised at a lower pH. Therefore, the fusion of autophagosomes with lysosomes results in the loss of yellow fluorescence and the appearance of only red fluorescence of RFP, indicating the completion of the autophagy process (Sinha et al., 2015). In DMSO-treated Vero E6 cells, a predominantly yellow colour was observed, indicating the merging of EGFP and RFP. However, in 3-indoleacetonitrile-treated Vero E6 cells, many autophagosomes predomin-

antly displayed yellow fluorescence (Fig. 6H), indicating that the autophagy process was prevented.

3.7. 3-Indoleacetonitrile inhibits the interaction of MAVS with the selective autophagy receptor SQSTM1

As mentioned above, autophagic flux was disturbed and MAVS protein levels were increased after 3-indoleacetonitrile treatment. Thus, we speculated that 3-indoleacetonitrile might inhibit the selective autophagic degradation of MAVS. Selective autophagy delivers intracellular material to lysosomes for degradation via selective autophagy–receptor interactions (Xu et al., 2018; Jo et al., 2020). To explore whether the autophagy receptor protein SQSTM1 mediates the degra-
dulation (accumulation) of MAVS by 3-indoleacetonitrile, we transfected Flag-MAVS plasmid into 293T cells, treated them with DMSO or 3-indoleacetonitrile, and detected the interaction between SQSTM1 and MAVS using a Co-IP assay. The results showed that the interaction of SQSTM1 with MAVS was obviously reduced after 3-indoleacetonitrile treatment. To further verify this, 293T cells were transfected with HA-SQSTM1 plasmid. Anti-HA immunoprecipitates were immunoblotted with anti-MAVS or anti-HA (SQSTM1) antibodies. These results suggested that 3-indoleacetonitrile attenuated the interaction between MAVS and SQSTM1. Therefore, these findings indicate that 3-indoleacetonitrile might interfere with autophagic flux by inhibiting the binding of MAVS to the selective autophagy receptor SQSTM1 and inhibiting the degradation of MAVS, resulting in the increase in expression of down-

stream interferons.

3.8. 3-Indoleacetonitrile-regulated interferon response is dependent on MAVS

To further validate the association between 3-indoleacetonitrile-
mediated interferon response and MAVS, we designed three pairs of siRNAs to target MAVS in 293T cells (The sequences of siRNAs were

injury and thickened alveolar septa were less severe in the SARS-CoV-2 infection-3-indoleacetonitrile-treated mice than in the SARS-CoV-2 infection + PBS-treated mice (Fig. 3E). The immunofluorescence results of the lung tissue were consistent with the viral loads in the lungs (Fig. 3E). Three mice in each group were sacrificed at 5 dpi, and their lungs, tracheas, and spleens were collected for viral load determination (Fig. 3F). The level of lung viral loads in the SARS-CoV-2 infection + 3-indoleacetonitrile treatment group remained significantly lower than those observed in mice in the PBS treatment group. The above findings indicate that 3-indoleacetonitrile might be a potential drug for treating SARS-CoV-2 infection in vivo.
Fig. 4. 3-indoleacetonitrile induces type I interferon production (A) 293T cells were co-transfected with an IFN-β reporter plasmid (pGL4–IFN-β), a Renilla luciferase plasmid (pRL-TK). Twenty-four hours after transfection, the cells were stimulated with different concentrations of 3-indoleacetonitrile (0 μM, 160 μM, 320 μM, 640 μM). Twelve hours after stimulation, the cells were collected for luciferase assay. (B) 293T cells were co-transfected with pGL4–IFN-β and pRL-TK plasmids. Sixteen hours after transfection, cells were stimulated with 680 μM 3-indoleacetonitrile. The cells were collected at different time points and subjected to luciferase assays. (C) Q-PCR analysis of IFN-β and ISGs mRNA in 293T cells stimulated with 680 μM 3-indoleacetonitrile for different time points. Data are presented as mean ± SD of three independent experiments. (D) 293T cells were infected with VSV virus (MOI = 0.1), treated with or without 3-indoleacetonitrile (680 μM) for 12 h, and subjected to qPCR analyses. (E) 293T cells were infected with HSV-1 (MOI = 0.1), treated with or without 3-indoleacetonitrile (680 μM) for 12 h, and subjected to qPCR analysis. Data are expressed as mean ± SD. Statistical analyses used two-way ANOVA test; *p < 0.05; **p < 0.01; ***p < 0.001.
listed in Table S1), which efficiency was detected by Western blot (Fig. 8A). The siRNA#1 had the ability to decrease MAVS by more than 80% on 293T cells (Fig. 8A). In parallel, effects of the siRNAs on cell viability were measured. Results showed that MAVS knockdown in 293T cells had a minimal effect on cell viability (Fig. 8B). Under this condition, MAVS knockdown cells or control cells were mock-infected or infected with SeV (a single-strand RNA murine parainfluenza virus which infects human cells and induces a robust antiviral interferon response) and were subsequently treated with or without 3-indoleacetonitrile for 12 h, followed by qPCR to detect the IFN-β mRNA levels. The results showed that 3-indoleacetonitrile significantly promoted IFN-β in siNC cells. However, it did not significantly promote IFN-β in MAVS knockdown cells (Fig. 8C), which suggested that MAVS played an important role in 3-indoleacetonitrile-mediated interferon response.

In addition, we investigated the antiviral effect of 3-indoleacetonitrile on wild-type (siNC) and MAVS knockdown (siMAVS) cells. As expected, the ability of 3-indoleacetonitrile to inhibit HSV-1 proliferation was obviously weakened in MAVS knockdown cells (Fig. 8D and E). Taken together, these data suggested that the broad-spectrum antiviral effect of 3-indoleacetonitrile is importantly associated with MAVS.

4. Discussion

Although the SARS-CoV-2 outbreak has largely been contained through public health measures, the potential for future outbreaks highlights the need for safe and effective therapies to combat viral infections. Compounds generated in nature have shown great promise in treating this disease because of their potential inhibitory properties and relatively low cytotoxicity (Jan et al., 2021). In addition, the antiviral effects of small molecule compounds depend on two factors. One is virus targeting, such as the current widely used anti-influenza drug oseltamivir, which blocks neuraminidase (NA) activity and, thus, virus release (Moscona, 2005). Another aspect of antiviral activity is host targeting. For example, PF-07321332, which targets 3CL protease, an enzyme necessary for the replication of SARS-CoV-2 viral activity is host targeting. For example, PF-07321332, which targets 3CL protease, an enzyme necessary for the replication of SARS-CoV-2, has shown great promise in treating this disease because of its potential inhibitory properties and relatively low cytotoxicity (Jan et al., 2021). Here, we report the antiviral activity against SARS-CoV-2 of 3-indoleacetonitrile, which efficiency was detected by Western blot (Fig. 8A). The siRNA#1 had the ability to decrease MAVS by more than 80% on 293T cells (Fig. 8A). In parallel, effects of the siRNAs on cell viability were measured. Results showed that MAVS knockdown in 293T cells had a minimal effect on cell viability (Fig. 8B). Under this condition, MAVS knockdown cells or control cells were mock-infected or infected with SeV (a single-strand RNA murine parainfluenza virus which infects human cells and induces a robust antiviral interferon response) and were subsequently treated with or without 3-indoleacetonitrile for 12 h, followed by qPCR to detect the IFN-β mRNA levels. The results showed that 3-indoleacetonitrile significantly promoted IFN-β in siNC cells. However, it did not significantly promote IFN-β in MAVS knockdown cells (Fig. 8C), which suggested that MAVS played an important role in 3-indoleacetonitrile-mediated interferon response.

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Several studies have demonstrated that indole derivatives have great potential to restrict viral infections. For example, our previous studies have demonstrated that 3-indoleacetonitrile impedes the replication of various subtypes of influenza viruses in vivo and in vitro (Zhao et al., 2021). However, the underlying molecular mechanism remains unclear. In general, the antiviral effects of small molecule compounds depend on two factors. One is virus targeting, such as the current widely used anti-influenza drug oseltamivir, which blocks neuraminidase (NA) activity and, thus, virus release (Moscona, 2005). Another aspect of antiviral activity is host targeting. For example, PF-07321332, which targets 3CL protease, an enzyme necessary for the replication of SARS-CoV-2 (Boras et al., 2021). In addition, the antiviral effect is achieved by regulating the host’s antiviral immune response; for example, the only approved hepatitis C virus (HCV) treatment for children aged <12 years is pegylated interferon plus ribavirin (Rosenthal et al., 2020); the small molecule GS-5734 (remdesivir) against Ebola and SARS-CoV-2 (Warren et al., 2016; Williamson et al., 2020). Host-targeted small molecule compounds are often resistant to a variety of viruses. In our study, 3-indoleacetonitrile was not only effective against HSV-1 (a double-stranded DNA virus) but also against VSV (a negative-strand RNA virus). Therefore, we focused on host targeting when exploring its antiviral mechanism. The interferon pathway plays an important antiviral role during virus infection (Liang et al., 2018). During viral infection, viral dsRNAs or dsDNAs are recognised by host pattern recognition receptors (PRRs) in mammalian cells (Zevini et al., 2017). Cytosolic DNA (viral DNA) and viral RNA are detected by cGAS and RIG-I-like receptors, respectively, and activate a signalling cascade that culminates in phosphorylation and subsequent nuclear translocation and DNA-binding of IRF3 to activate IFN-β transcription (Banerjee et al., 2014). Previous studies have demonstrated that 293T cells do not express endogenous cGAS or STING (Stempel et al., 2019). In this study, 3-indoleacetonitrile-triggered interferon production in 293T cells. Therefore, we excluded endogenous STING and targeted the RIG-I-MAVS signalling pathway for further exploration.

MAVS has been identified as an essential adaptor protein in cytosolic nucleic acid-sensing pathways that promotes the production of type I interferon during virus infection ( Seth et al., 2005). A number of viruses evade the host immune response by perturbing MAVS. For example, the P81-F2 protein of influenza A virus inhibits type I interferon production by interacting with MAVS (Liu et al., 2021a). The HSV-1 US11 protein competes with RIG-I/MDA5 for RNA binding via the RNA-binding domain at the C-terminal end, hindering signal transmission to downstream MAVS, thereby inhibiting the production of type I interferon (Xing et al., 2012). SARS-CoV-2 M inhibits type I interferon production by inducing selective mitochondrial autophagy to degrade MAVS (Hui et al., 2021). The downstream region of MAVS is divided into the NF-κB and interferon signalling pathways. Nuclear import of p65 triggers the transcription of NF-κB target genes (Liu et al., 2020), and nuclear import of IRF3 triggers type I interferon production (Johnson et al., 2018). In the present study, 3-indoleacetonitrile treatment induced phosphorylation of p65 and IRF3, as well as nuclear import. This indicates that the role of 3-indoleacetonitrile lies in the intersection and above stages of the NF-κB and IFN signalling pathways, that is, at or upstream of the MAVS level in the RIG-I signalling axis. Notably, we found that 3-indoleacetonitrile induced an increase in MAVS protein expression and may be related to autophagy. SQSTM1/p62 is a well-known selective autophagy substrate that functions as a selective autophagy receptor. Specifically, SQSTM1 binds to its targets and LC3 protein, which results in the autophagic degradation of SQSTM1 as well as its binding targets (Jo et al., 2020; Ichimura et al., 2008; Kirkin et al., 2009). Previous studies have shown that HFE (a homeostatic iron regulator) binds to MAVS and promotes SQSTM1-mediated degradation of MAVS via the selective autophagy-lysosome pathway, leading to a suppressed antiviral immune response (Liu et al., 2021b). In the present study, 3-indoleacetonitrile treatment interfered with the interaction of SQSTM1 with MAVS. This could explain why 3-indoleacetonitrile induced an increase in MAVS protein accumulation, that is, it inhibited the selective autophagic
3-indoleacetonitrile treatment promoted the protein level of MAVS and interfered with the completion of autophagy flux in cells. (A) 293T cells were transfected with the indicated plasmids, and a reporter assay was conducted after mock treatment or treatment with 3-indoleacetonitrile. (B) 293T cells were transfected with indicated plasmids and mock treated or treated with 3-indoleacetonitrile and analysed for MAVS ubiquitylation via western blotting. (C) A549 cells were transfected with the indicated plasmids and mock treated or treated with 3-indoleacetonitrile and analysed for MAVS ubiquitylation via western blotting. (D) 293T cells were mock treated or treated with 3-indoleacetonitrile, lysates were analysed using immunoblotting. (E) A549 cells were mock treated or treated with 3-indoleacetonitrile, lysates were analysed using qPCR after 12 h. (F) Caco-2 cells were mock treated or treated with different concentrations of 3-indoleacetonitrile for 16 h, lysates were analysed using qPCR. (G) GFP-LC3 dot formation in Vero-E6 cells transiently transfected with GFP-LC3 and either left untreated (DMSO) or treated with 3-indoleacetonitrile for 12 h, or treated with CQ for 4 h. Scale bar, 10 μm. (H) Vero-E6 cells were transfected with RFP-GFP-LC3 for 24 h and then were mock treated or treated with 3-indoleacetonitrile or treated with CQ or starved in EBSS medium for 2 h, and then analysed for autophagosome. Scale bar, 10 μm. Data are expressed as mean ± SD. Statistical analyses were conducted using Student’s t-test; *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 6. 3-indoleacetonitrile treatment promoted the protein level of MAVS and interfered with the completion of autophagy flux in cells. (A) 293T cells were transfected with the indicated plasmids, and a reporter assay was conducted after mock treatment or treatment with 3-indoleacetonitrile. (B) 293T cells were transfected with indicated plasmids and mock treated or treated with 3-indoleacetonitrile and analysed for MAVS ubiquitylation via western blotting. (C) A549 cells were mock treated or treated with 3-indoleacetonitrile, lysates were analysed using immunoblotting. (D) 293T cells were mock treated or treated with 3-indoleacetonitrile, lysates were analysed using immunoblotting. Error bars, mean ± SD of three experiments. (E) A549 cells were mock treated or treated with 3-indoleacetonitrile, lysates were analysed using qPCR after 12 h. (F) Caco-2 cells were mock treated or treated with different concentrations of 3-indoleacetonitrile for 16 h, lysates were analysed using immunoblotting. (G) GFP-LC3 dot formation in Vero-E6 cells transiently transfected with GFP-LC3 and either left untreated (DMSO) or treated with 3-indoleacetonitrile for 12 h, or treated with CQ for 4 h. Scale bar, 10 μm. (H) Vero-E6 cells were transfected with RFP-GFP-LC3 for 24 h and then were mock treated or treated with 3-indoleacetonitrile or treated with CQ or starved in EBSS medium for 2 h, and then analysed for autophagosome. Scale bar, 10 μm. Data are expressed as mean ± SD. Statistical analyses were conducted using Student’s t-test; *p < 0.05; **p < 0.01; ***p < 0.001.

Yuen et al. demonstrated that SARS-CoV-2 induces very weak IFN expression in infected cells (Chu et al., 2020). The absence of IFN production likely hampers the early innate immune response to SARS-CoV-2 infection. Previous studies reported the activity of type I interferons against SARS-CoV-2 infection in vitro, and the results were encouraging. Hoagland et al. presented compelling evidence that IN administration or induction of IFN-1 provides protection against SARS-CoV-2 infection, whether for prophylaxis or for early therapeutic use (Hoagland et al., 2021). In the present study, 3-indoleacetonitrile, a small molecule that has been shown to stimulate interferons, exhibited effective antiviral activity in vitro and in vivo. Thus, our results have potential implications in the development of effective treatment strategies for COVID-19. Approximately 80% of patients display mild symptoms after infection with SARS-CoV-2 (Shen et al., 2020). Deaths in severe and critically ill patients are mostly due to heart, kidney, and liver failure, and the main cause of damage to these organs is a virus-induced cytokine storm. Here, we demonstrated that 3-indoleacetonitrile promotes the elevation of interferons and was accompanied by the activation of the NF-κB signalling pathway. The inflammatory response is a double-edged sword in host defence and immune responses against SARS-CoV-2 infection. Therefore, potential effects that 3-indoleacetonitrile has on active inflammatory response should be paid attention to in the treatment of viral infections.

Indole, also known as benzopyrrole, is a bicyclic structure formed by a benzene ring fused with a pyrrole ring. Indole is reportedly a key component of many bioactive molecules’ skeleton as well as an important part of the molecular structure of some drugs (e.g. antioxidants (Ahuja and Siddiqui, 2014), antiviral drugs (Zhang et al., 2015), antidepressant (Zhou et al., 2008), etc.). Moreover, the antiviral effect of 3-indoleacetonitrile may be due to its indole group activity. Our previous study suggested that the small molecules, indole-3-carboxaldehyde and arbidol, which have the same indole group with 3-indoleacetonitrile, also showed some antiviral activity (Zhao et al., 2021). The widely use of indole in medical field not only promotes the development of many drugs, but also shows good effects in treating many kinds of diseases.
of its synthetic methods, but also will attract more and more researchers
to develop new drugs based on the indole skeleton.

In summary, we demonstrated that 3-indoleacetonitrile exhibited
potential inhibitory effects against SARS-CoV-2 infection both in vitro
and in vivo, which may contribute to the development of new broad-
spectrum antiviral activity as therapeutic agents for COVID-19.

Author contributions

XH, XY, KH, YZ, LL, YJ, and MJ conceived and designed the exper-
iments. XH, XY, LH, TX, and YZ performed the experiments. XH
analysed the data. KH, CL, YZ, and FS contributed to the reagents and
materials. XH and MJ wrote the paper. All authors contributed to the
article and approved the submitted version.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2022.105465.

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