Novel Fatty Acid in Cordyceps Suppresses Influenza A (H1N1) Virus-Induced Proinflammatory Response Through Regulating Innate Signaling Pathways

Run-Feng Li,*, Xiao-Bo Zhou, Hong-Xia Zhou, Zi-Feng Yang, Hai-Ming Jiang, Xiao Wu, Wen-Jia Li, Jian-Jian Qiu, Jia-Ning Mi, Ming Chen, Nan-Shan Zhong, Guo-Yuan Zhu, and Zhi-Hong Jiang

ABSTRACT: Influenza virus (IV) infections usually cause acute lung injury characterized by exaggerated proinflammatory responses. The paucity of therapeutic strategies that target host immune response to attenuate lung injury poses a substantial challenge in management of IV infections. In this study, we chemically synthesized a novel fatty acid (2Z,4E)-deca-2,4-dienoic acid (DDEA) identified from Chinese Cordyceps by using UHPLC–Q-TOF–MS techniques. The DDEA did not inhibit H1N1 virus replication but attenuated proinflammatory responses by reducing mRNA and protein levels of TNF-α, IFN-α, IFN-β, IL-6, CXCL-8/IL-8, CCL-2/MCP-1, CXCL-10/IP-10, CCL-3/MIP-1α, and CCL-4/MIP-1β in A549 cells and U937-derived macrophages. The anti-inflammatory effect occurred through downregulations of TLR-3-, RIG-I-, and type I IFN-activated innate immune signaling pathways. Altogether, our results indicate that DDEA may potentially be used as an anti-inflammatory therapy for the treatment of IV infections.

1. INTRODUCTION

Influenza virus (IV) is a segmented negative-sense single-stranded RNA virus that represents a great burden on public health. The most common circulating subtypes are influenza A (H1N1) and A (H3N2) viruses that cause seasonal epidemic with 250,000 to 500,000 deaths annually.1 Because of the ability to undergo adaptation, zoonotic influenza viruses such as avian influenza H5N1 and H7N9 viruses can occasionally cross the species barrier from poultry to humans, causing more severe disease than seasonal IVs.2,3 The burden of IV infection requires the use of antiviral medications to reduce viral shedding and relieve symptoms. At present, two classes of influenza antivirals are available for clinical use: neuraminidase inhibitors (i.e., oseltamivir, zanamivir, and peramivir) and cap-dependent endonuclease inhibitor (i.e., baloxavir marboxil).4 However, delay in diagnostic and therapeutic procedures and development of virus resistance can lead to reduced benefits for patients.5–7

It is widely accepted that not only virus replication competence but also host immune response contribute to disease outcome in IV disease.8 Early recognition of IVs is mediated by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and retinoic acid inducible gene-I (RIG-1) in respiratory epithelial cells and alveolar macrophages.9–11 Activation of these molecules triggers signal transduction pathways, leading to the production of proinflammatory cytokines and the subsequent clearance of the virus.11 In an otherwise healthy individual, IV causes mild infections, and virus clearance can be accomplished without causing damage in the respiratory tract.12 However, IV infections caused by pandemic or highly pathogenic strains can lead to fulminant viral pneumonia in a high-risk population, including infants, young children, pregnant women, or the elderly, who progress to acute respiratory distress syndrome (ARDS) or even die.13,14 It has been shown that cytokine storms, characterized by robust secretion of proinflammatory cytokines, such as interleukin (IL)-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), interferon gamma-induced protein 10 (IP-10), tumor necrosis factor-α (TNF-α), and C–C motif chemokine ligand (CCL)-5, played a key role in the pathogenesis of severe IV infection.15,16 These findings highlight the need for novel therapeutic options to dampen adverse host immune responses.
Chinese Cordyceps (CS) is a famous and precious herb that has been widely used as a traditional Chinese medicine with immunomodulatory, metabolic, and tonic activity.\(^\text{17,18}\) CS mainly consists of the stroma of the fungus *Cordyceps sinensis* (Berk.) Sacc. (family Hypocreaceae) and the dead caterpillar of *Hepialus armoricanus* (family Hepialidae). *Ophiocordyceps* sinensis, the anamorph of CS, has been cultured as an alternative of the wild CS and exhibited similar pharmacological activities.\(^\text{19,20}\) Multiple chemical constituents, such as nucleosides, amino acids, cyclopeptides, fatty acids, saccharides, mannitol, and sterols, have been isolated from this fungus.\(^\text{21,22}\) In our previous work, numerous sphingolipids in the wild CS and their mycelia were comprehensively profiled by using the sphingolipidomic approach established in our laboratory.\(^\text{23−25}\) In a continuous study, (2Z,4E)-deca-2,4-dienoic acid (DDEA) (Figure 1A) was isolated from *O*. *sinensis* for the first time and elucidated by means of HRMS, 1H NMR, and 13C NMR spectroscopic analysis. Furthermore, UHPLC−MS/MS analysis showed that this fatty acid also existed in the wild CS. To confirm its structure, DDEA was synthesized from (2E)-2-octenal by using the Still−Gennari modification of the Horner−Wadsworth−Emmons olefination as described.\(^\text{26,27}\) Herein, we demonstrated the inhibitory effect of DDEA on influenza A (H1N1)−induced cytokine expressions in vitro and deciphered possible mechanisms underlying the anti-inflammatory effect of DDEA on PRR- and IFN-dependent signaling pathways.

2. RESULTS

2.1. Chemistry. DDEA was isolated as a colorless oil from *O. sinensis*. Its molecular formula was established to be C\(_{10}\)H\(_{16}\)O\(_2\) from the HR−ESI−MS ion peak at m/z 169.1217 [M + H]\(^+\) (calcld [M + H]\(^+\) 169.1223), indicating that DDEA has three degrees of unsaturation. The 1H NMR data indicate the presence of one methyl group at \(\delta_H 0.88\) (3H, t, \(J = 6.6\) Hz), four methylene groups at \(\delta_H 2.14\) (2H, q), \(1.38−1.48\) (2H, m), and \(1.28−1.39\) (4H, m), and four olefinic groups at \(\delta_H 7.35\) (1H, ddd, \(J = 11.3, 15.0, 1.0\) Hz, H-4), \(6.66\) (1H, t, \(J = 11.3\) Hz, H-3), \(6.11\) (1H, dt, \(J = 15.0, 7.5\) Hz, H-S), and \(5.57\) (1H, d, \(J = 11.3\) Hz, H-2) (Figure S1). The 13C NMR data revealed 10 carbon signals, corresponding to one methyl carbon (\(\delta_C 14.0\)), four methylene carbons (\(\delta_C 33.0, 31.5, 28.5,\) and \(22.5\)), four olefinic carbons (\(\delta_C 147.7, 147.2, 127.0,\) and \(114.4\)), and one carbonyl carbon (\(\delta_C 171.6\)) (Figure S2). Configuration assignment was based on the multiplicities and vicinal coupling constants of the compound from 1H NMR spectrum. The signals at \(\delta_H 5.57\) (1H, d, \(J = 11.3\) Hz, H-2) and \(\delta_H 6.66\) (1H, t, \(J = 11.3\) Hz, H-3) were assigned to two olefinic protons of a double bond with Z geometry, and the proton at \(\delta_H 5.57\) (d, \(J = 11.3\) Hz) was assigned to the \(\alpha\) position of carbonic acid. The coupling constant (\(J = 15.0\) Hz) of \(\delta_H 3.45\) (1H, d, \(J = 11.3\) Hz, H-2) and \(\delta_H 6.66\) (1H, t, \(J = 11.3\) Hz, H-3) were assigned to two olefinic protons of a double bond with Z geometry, and the proton at \(\delta_H 5.57\) (d, \(J = 11.3\) Hz) was assigned to the \(\alpha\) position of carbonic acid. The coupling constant (\(J = 15.0\) Hz) of \(\delta_H 7.35\) (1H, d, \(J = 15.0, 11.3, 1.0\) Hz, H-4) and \(\delta_H 6.11\) (1H, dt, \(J = 15.0, 7.5\) Hz, H-S) clearly indicated an E geometry for the double bond.

To confirm its structure and afford enough samples for bioactivity evaluation, DDEA was synthesized from (2E)-2-octenal, as shown in Scheme 1. The unsaturated (2E)-2-
octenal was converted to (2Z,4E)-conjugated ester by using the Still-Gennari modification of the Horner−Wadsworth−Emmons olefination. Following hydrolysis of the conjugated ester was performed by lithium hydroxide in ethanol and tetrahydrofuran to afford DDEA. Its HRMS, 1H NMR, and 13C NMR spectrum are in accordance with the spectra of the compound isolated from O. sinensis.

We next performed UHPLC−MS/MS analysis to confirm the presence of DDEA in CS. Comparison of chromatographic retention time showed that a peak of CS has the same RT (RT = 17.18 min) value as with DDEA (Figure 1B). Then, the compound was confirmed by comparing the high-resolution MS and MS/MS spectra with DDEA. Results showed that high-resolution MS (m/z 169.12) (Figure 1C) and fragment ions (such as m/z 151.1, 133.1, 123.1, 109.1, 95.1, 81.1, and 67.1) (Figure 1D) are consistent with those of DDEA. Therefore, we prove that DDEA is present in the CS.

2.2. Cytotoxicity and Antiviral Activity of DDEA in A549 Cells and U937-Derived Macrophages. The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay was employed to evaluate the cytotoxicity in A549 cells and U937 macrophages. The results showed that DDEA did not cause cytotoxicity at the concentration up to 100 μM with median toxic concentration (TC50) values found to be 190.7 and 155.8 μM for A549 cells and U937 macrophages, respectively (Figure 2A,B). The anti-influenza activity of DDEA was then examined in MDCK cells by using CPE reduction assay and further quantified by MTT staining. The DDEA did not reduce the PR8-induced CPE or the cell viability loss detected by MTT with the median inhibitory concentration (IC50) > 100 μM (data not shown). We next measured the infectious virus in A549 cell culture supernatants by the 50% tissue culture infective dose (TCID50) assay. No significant difference in the virus titer was observed in infected cells treated with or without DDEA (Figure 2C,D). These results suggest that DDEA may not inhibit IV replication.

2.3. Inhibitory Effect of DDEA on Proinflammatory Cytokines. To understand the inhibitory effect of DDEA on proinflammatory responses, we measured mRNA and corresponding protein levels of various cytokines in PR8-infected A549 cells and U937 macrophages. The mRNA and protein levels of TNF-α, IFN-α, IFN-β, IL-6, CXC chemokine ligands (CXCL)-8/IL-8, CCL-2/MCP-1, CXCL-10/IP-10, CCL-3/macrophage inflammatory protein (MIP)-1α, CCL-4/MIP-1β were upregulated in both cells at 24 h postinfection (hpi). At both levels, DDEA showed a significant inhibition on PR8-induced cytokine production in a dose-dependent manner in A549 cells (Figure 3A,B). Similar trends were also observed in PR8-infected U937 macrophages with the treatment of DDEA (Figure 4A,B). These results suggest that DDEA exhibits potent anti-inflammatory effect against H1N1 infection.

Figure 2. Cytotoxicity and antiviral effect of DDEA in A549 cells and U937 macrophages. For cytotoxicity assay, A549 cells (A) and U937 macrophages (B) were incubated with twofold serial dilution of DDEA for 48 h, followed by MTT staining. The absorbance at a wavelength of 490 nm was measured. In the TCID50 assay, A549 cells (C) and U937 macrophages (D) were infected by A/Puerto Rico/8/34 (H1N1) (PR8) virus at a multiplicity of infection (MOI) of 1 with indicated concentrations of DDEA. Viral replication in culture supernatants was determined at 24 h postinfection (hpi). Results are presented as the mean ± SD from three independent experiments.
2.4. Effect of DDEA on H1N1-Induced Innate Cellular Signaling Pathway. Because PRR-mediated signaling pathways regulate cytokine expressions, we next investigated whether the inhibitory effect of DDEA on cytokine levels would be related to its impact on PRR pathways. As expected, DDEA treatment significantly decreased the levels of TLR-3 and RIG-I in PR8-infected A549 cells (Figure 5A,B). Also, phosphorylation of the downstream interferon regulatory factors 3 (IRF-3) and p65 was also markedly downregulated by DDEA (Figure 5C,D). Because we found that DDEA...
attenuated the type I IFN expression, we speculated that the phosphorylation of IFN signaling molecules may also be affected. Accordingly, we found that the phosphorylated signal transducer and activator of transcription 2 (STAT2) was also remarkably inhibited by DDEA (Figure 5E). The DDEA at doses of 25 and 50 μM inhibited these host sensors at similar levels, suggestive of an unapparent dose-dependent manner.

3. DISCUSSION

The CS contains plenty of saturated and polyunsaturated fatty acids (PUFA). Guo et al. reported 17 fatty acids and their
relative and absolute contents via gas chromatography–mass spectrometry (GC–MS) with C16:0, C18:0, C18:1, C18:2, and C18:3 as major constituents in natural O. sinensis.28 In another study, Guo’s group compared fatty acids’ contents between wild and cultured CS.29 In addition, Yang et al. reported 10 fatty acids, namely, lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid, and lignoceric acid in natural and cultured CS using pressurized liquid extraction, trimethylsilyl derivatization, and GC–MS analysis.30 The CS extract and the acidic polysaccharide isolated from Cordyceps militaris have been proven effective in alleviating disease severity in IV-infected mice by modulating immune functions.31,32 Besides, many other compounds with anti-inflammatory or antiviral properties, such as cordycepin, nucleosides, cyclopeptides, sterols, sphingolipids, and fatty acids, are also present in Cordyceps species.31,32 It has been shown that PUFA inhibited IV replication and exhibited pharmacological activity in innate and adaptive antiviral responses, as well as inflammation control.33 In this study, we chemically synthesized a conjugated PUFA DDEA identified from CS and found that DDEA did not inhibit H1N1 virus replication but attenuated proinflammatory responses by reducing levels of a number of cytokines. Additionally, DDEA inhibited activations of TLR-3, RIG-I, and type I IFN-activated signaling pathways.

Highly pathogenic IVs such as 1918 H1N1 and avian H5N1 and H7N9 viruses often cause an inflammatory cytokine storm that is responsible for the development of severe pneumonia, ARDS, or even death. Blockage of cytokine induction is the most direct tactic to blunt the cytokine storm. Aberrant cytokine responses can also be mitigated by inhibitors targeting components in innate immune signaling, such as antagonists of PRRs and kinases.34,35 In the present study, we found that DDEA did not suppress virus replication in lung epithelial cells and macrophages, the primary targets for IV, nor did it inhibit the phosphorylation of extracellular signal-regulated kinase (ERK), protein kinase B (PKB, Akt), and p38 used by IV to facilitate its entry, RNP translocation, and vRNA synthesis.36,37 Following IV infection, the viral ssRNA is sensed by TLR family members, such as TLR-3, -7, and -8, and RIG-I like receptors, such as RIG-I and melanoma differentiation-associated gene 5 (MDA5).38 Recognition by these PRRs leads to the activation of nuclear factor-κB (NF-κB) and IRF-3, resulting in the induction of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFNs.38 In an appropriate immune response, expressions of proinflammatory cytokines facilitate viral clearance, which was followed by a resolution of inflammation, tissue repair and a return to homeostasis.39 However, in severe influenza cases, hyperactivated innate signaling cascades orchestrate uncontrolled inflammatory responses, contributing to severe lung tissue injury.40,41 Previous studies found that administration of TLR4 antagonists reduced levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and keratinocyte chemoattractant (KC) in
mice challenged with lethal IV. Li et al. recently demonstrated that polysaccharides isolated from Radix isatidis inhibited IL-6 and CCL-5 expressions by blocking upstream TLR3 in vitro. Small interference RNA (siRNA)-mediated RIG-I silencing resulted in significant decreased levels of TNF-α, IFN-β, IFN-λ1, and IP-10 in H5N1- and H1N1-infected human macrophages. These findings indicate that TLRs and RIG-I are required for inductions of proinflammatory cytokines. Comparably, we found that the downregulation of TLR-3, RIG-I, and the downstream p-IRF-3 and p-p65 by DDEA resulted in blunted cytokine responses, which suggests that DDEA may serve as a potential immunomodulator by regulating TLR-3- and RIG-I-dependent signaling pathways.

The type I (IFN-α, β), II (IFN-γ), and III (IFN-λ1−3) IFNs are well-known cytokines essential to limit the replication and dissemination of IVs. The type I IFN induction can be completely shut down after dual knockdown of RIG-I and TLR-3, indicating that TLR-3 and RIG-I contribute to the maximum IFN production following IV infection. Therefore, the decrease in levels of type I IFNs by DDEA may be the consequence of inhibitions of TLR-3 and RIG-I. The type I IFN induced by PPRs binds to the interferon α and β receptor subunit 1 (IFNAR1)/IFNAR2 receptor, which initiates the formation of the interferon-stimulated gene factor 3 (ISGF3) complex comprising IRF9, p-STAT1, and p-STAT2. The ISGF3 is then translocated to the nucleus and drives the transcription of various antiviral genes, namely, IFN-stimulated genes (ISGs), such as the Mx family, interferon-induced transmembrane protein family (IFITMs), cholesterol 25-hydroxylase (CH25H), and the tripartite motif-containing proteins (TRIM). Although we found that the IFNs and p-STAT2 were downregulated with DDEA treatment, we did not observe a significant increase in viral replication (Figure 4). It is possible that the impaired IFN/STAT signaling induced by DDEA may delay the clearance of virus. Therefore, combination of DDEA and an antiviral agent, rather than DDEA alone, may be a better therapeutic strategy for treating IV infection. In addition, the type I IFN response not only induces an important antiviral state but also activates various proinflammatory pathways mediated by phosphoinositide 3-kinase (PI3K), NF-κB, or mitogen-activated protein kinase (MAPK). An in vivo study reported that although IFN-α treatment restricted IV replication, it exacerbated disease by inducing proinflammatory cytokine production and promoting innate cell recruitment. It is conceivable that the inhibition of type I IFN by DDEA may help in reducing such adverse immune consequences.

In conclusion, we demonstrate that DDEA inhibits proinflammatory cytokine expressions by interrupting TLR-3, RIG-I, and IFN-signaling pathways activated by IV (Figure 6). The results of this study indicate that DDEA may potentially be used as an anti-inflammatory therapy for the treatment of IV infections.
4. MATERIALS AND METHODS

4.1. Chemicals and Materials. Solvents and reagents were obtained from a commercial supplier. For simplicity, (2E)-2-octenal, lithium bis(trimethylsilyl)amide (LiHMDS), trimethyl phosphonoacetate, bis(2,2,2-trifluoroethoxy) (methoxycarbonylmethyl) phosphonate, chloroform, tetrahydrofuran, dichloromethane, ethyl acetate (EtOAc), hydrochloric acid, methanol (MeOH), sodium bicarbonate (NaHCO3), lithium hydroxide (LiOH), trimethylamine, ammonium chloride (NH4Cl), sodium chloride (NaCl), magnesium sulfate (MgSO4), lithium hydroxide (LiOH), trimethylamine, n-hexane, petroleum ether, and acetonitrile (ACN) were purchased from TCI or 90pingchem (Shanghai, China). NMR solvent CDCl3 was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). *Pacilomyces hepiali* and CS were purchased from commercial sources. Deionized water was prepared in our laboratory by a Millipore Milli-Q plus system (Millipore, Bedford, MA, USA). Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Ascend 600 NMR spectrometer (Bruker, Zurich, Switzerland) at 600 MHz for 1H NMR and 150 MHz for 13C NMR. HRMS and LC-MS/MS spectrum were obtained on an Agilent 6550 Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) in positive ion mode. Thin layer chromatography (TLC) was performed on silica gel 60 F254 plates and RP-18 F254 plates (200 μm thick, Merck KGaA, Germany). Column chromatography was conducted with Davisil silica gel (40–63 μM, Grace, Columbia, MD, USA) and Waters ODS (55–105 μM, Miford, MA, Ireland).

4.2. Cell Lines and Virus. Human lung carcinoma A549 cells, human leukemia U937 cells, and Madin-Darby canine kidney (MDCK) cells were purchased from American Type Culture Collection (ATCC). The A549 and MDCK cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) at 37 °C. The U937 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2.5 mM glutamine, and 10 mM HEPES at 37 °C and differentiated into macrophage-like cells in the presence of 100 nM of phorbol myristate acetate (PMA) (Sigma) for 48 h. Influenza H1N1 virus PR8 strain, A/Puerto Rico/8/34, was purchased from ATCC, propagated in the allantoic cavities of chicken embryos, concentrated in a rotary evaporator under reduced pressure to 30 mL, and the concentrate was subjected to centrifugation at 15,000 g at 4 °C for 30 min; the combined solutions were concentrated in a rotary evaporator under reduced pressure to 30 μL. Then, 10 μL of the sample was injected into an Agilent Zorbax Eclipse Plus C18 (1.8 μm, 2.1 × 150 mm) column on an Agilent 6550 ultra-high-performance liquid chromatography–ultrahigh definition-quadrupole time-of-flight mass spectrometry (UHPLC–UHD-Q-TOF–MS). The mobile phase was composed of 1% formic acid (FA) in water (A) and 1% FA in ACN (B), and the gradient elution was performed at a flow rate of 0.35 mL/min as follows: 5% B for 3 min, 5–68% B over 20 min, and 100% B for 5 min. The dry-gas (N2) temperature and flow rate were 325 °C and 9 L/min, respectively. The LC/MS data of unknown compound were analyzed based on peak retention time, accurate mass, and fragment ions. The MS spectra were acquired in the positive mode.

4.3. Extraction and Isolation of DDEA. The dried powder of *P. hepiali* (166.6 g) was extracted with chloroform three times in an ultrasonic bath. The combined solutions were concentrated in a rotary evaporator under reduced pressure to afford a concentrate (30 mL). The concentrate was subjected to a silica gel column eluted with n-hexane–ethyl acetate (95:5 → 0:100, v/v), followed by chloroform–methanol–water (9:1:0.1 → 8:2:0.2, v/v) to yield 38 fractions. Fraction 5 (1.9 g) was further separated by silica gel column chromatography using petroleum ether–ethyl acetate (95:5 → 0:100, v/v) to give 12 subfractions. Fraction 5–5 (0.9 g) was rechromatographed on silica gel (n-hexane–ethyl acetate, 94:6 → 50:50, v/v) to give fraction 5–5–4 (100 mg). Fraction 5–5–4 was further subjected to silica gel column chromatography eluted with petroleum ether–ethyl acetate (94:6 → 50:50, v/v) to afford fraction 5–5–4–1 (57 mg). The last subfraction was purified by ODS column chromatography (methanol–water, 70:30 → 100:0, v/v) to give DDEA (9 mg). 1H NMR (600 MHz, CDCl3): δ 7.35 (d, J = 11.3 Hz, 1H, H-2), 2.17 (g, 2H), 1.40–1.45 (m, 2H), 1.28–1.32 (m, 4H), 0.88 (t, J = 6.9 Hz, 3H); 13C NMR (150 MHz, CDCl3): δ 171.6, 147.7, 147.2, 127.0, 114.4, 33.0, 31.5, 28.5, 22.5, 14.1. HRMS-ESI (m/z); [M + H]+ calc for [C9H13O3]5+, 169.1223; found, 169.1217.

4.4. Synthesis of DDEA. A solution of bis(2,2,2-trifluoroethoxy) (methoxycarbonylmethyl) phosphonate (1.6 g, 5.0 mmol) and 18-crown-6 (12.2 g, 46.5 mmol) in tetrahydrofuran (THF) (100 mL) at 0 °C was treated with a 1.3 M solution of lithium bis(trimethylsilyl)amide (LiHMDS) in THF (3.9 mL). The reaction mixture was stirred for 15 min before a solution of (2E)-2-octenal (0.6 mL, 4.0 mmol) was added at 0 °C. The reaction mixture was stirred for 2 h, saturated aqueous NH4Cl (40 mL) and saturated aqueous NaCl (80 mL) were added, and stirred for another 2 h. Then, the reaction mixture was extracted with EtOAc (3 × 50 mL), the organic layer was dried (MgSO4), filtered, and concentrated in vacuo. The residue was purified by flash silica gel chromatography (hexane/ethyl acetate 100:1, v/v) to give a colorless liquid. Then, a solution of methyl (2Z,4E)-deca-2,4-dienoate (126.3 mg, 0.7 mmol) in 33% THF–EtOH (6.0 mL) was treated with aqueous 0.8 M LiOH (4.0 mL), and the reaction mixture was stirred at 25 °C for 15 h. The reaction mixture was acidified with 12 M HCl and treated with water (50 mL) and extracted with EtOAc (3 × 50 mL). The organic layer was dried with MgSO4, filtered, and concentrated in vacuo. The residue was purified by flash silica gel chromatography (hexane/ethyl acetate 3:1, v/v) to give a colorless liquid (77.5 mg, 72%). Rf = 0.5 (hexane/ethyl acetate 3:1, v/v). Its HRMS, 1H NMR, and 13C NMR spectral data are in good agreement with those of the compound isolated from *O. sinensis*.

4.5. Detection of DDEA in the CS. Dried CS (0.3 g) was extracted with MeOH (2 mL) twice in an ultrasonic bath for 30 min; the combined solutions were concentrated in a pressure-blowing concentrator to afford a concentrate (250 μL). Then, 10 μL of the sample was injected into an Agilent Zorbax Eclipse Plus C18 (1.8 μm, 2.1 × 150 mm) column on an Agilent 6550 ultra-high-performance liquid chromatography–ultrahigh definition-quadrupole time-of-flight mass spectrometry (UHPLC–UHD-Q-TOF–MS). The mobile phase was composed of 1% formic acid (FA) in water (A) and 1% FA in ACN (B), and the gradient elution was performed at a flow rate of 0.35 mL/min as follows: 5% B for 3 min, 5–68% B over 20 min, and 100% B for 5 min. The dry-gas (N2) temperature and flow rate were 325 °C and 9 L/min, respectively. The LC/MS data of unknown compound were analyzed based on peak retention time, accurate mass, and fragment ions. The MS spectra were acquired in the positive mode.

4.6. Cytotoxicity Assay. The A549 and PMA-differentiated U937 cell monolayers were rinsed twice with PBS. After removal of the wash buffer, the twofold serially diluted DDEA was added to cells and incubated for 48 h. After removing the supernatant, cell culture plates underwent cytotoxicity assessment by the MTT assay as previously described. The TC50 was calculated by the GraphPad Prism 8.0 software.

4.7. Antiviral Assay. In the CPE reduction assay and MTT assay, the MDCK cell monolayers in 96-well plates were inoculated with PR8 at a MOI of 0.001 at 37 °C for 2 h. After the inoculum was removed, the cells were incubated with twofold serially diluted DDEA. After 72 h, the infected cells presented 100% cytopathogenic effect (CPE) under the
microscope. The percentage of CPE in infected cells with DDEA treatment was recorded. The medium was then removed and replaced by 1 mL of 0.5 mg/mL MTT solution. Following 4 h incubation, MTT was removed and 100 μL dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at a wavelength of 490 nm using a microplate reader (Synergy HT, Bio-Tek). The IC_{50} of DDEA was calculated by the GraphPad Prism 8.0 software.

In the virus titration experiment, confluent monolayers of A549 cells and U937 macrophages were rinsed twice with PBS and subsequently infected with PR8 at a MOI of 1 for 2 h at 37 °C. After virus adsorption, the inoculum was removed and supplemented with culture medium containing twofold serially diluted DDEA. After 24 h of incubation, supernatants were collected for viral titration using the TCID_{50} assay and cytokine measurement by the Bio-Plex assay, while cell layers were collected for RNA isolation.

4.8. RNA Isolation and Reverse Transcription Real-Time Quantitative PCR. Total RNA was isolated using FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme Biotech) according to the manufacturer’s protocol. cDNA was generated from total RNA using the PrimeScript RT-PCR Kit (Takara). Real-time quantitative PCR was performed with target gene-specific TaqMan probes and primers by using an Applied Biosystems 7500 system. Each primer and probe used in this study is listed in Supporting Information Table S1. Relative quantification of gene expression was calculated by the 2^{-ΔΔCT} method with GAPDH as the endogenous reference.

4.9. Proinflammatory Cytokine Measurement in the Culture Supernatant. Proinflammatory cytokine levels in cell culture supernatants were detected using Bio-Plex Pro Human Cytokine Screening Panel (Bio Rad) according to the manufacturer’s instructions. The test plate was analyzed using a Bio-Plex Luminex 200 XYP instrument (Bio-Rad Laboratories). The data were calculated using Bio-Plex Manager software (Version 5.0, Bio-Rad Laboratories).

4.10. Western Blot Analysis. The A549 cell monolayers in six-well plates were incubated with PR8 at a MOI of 1 for 2 h at 37 °C. The virus inoculum was then removed and replaced with DMEM medium containing twofold serially diluted DDEA. At 24 hpi, cells were lysed on ice for 10 min with radio immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing a phosphatase inhibitor cocktail (Sigma). Protein concentration was measured using a bicinecinic acid protein assay kit (Invitrogen Life Technologies). Western blots were performed on PVDF membranes as described, using primary antibodies (CST Biological Reagents) to identify TLR-3, RIG-I, IRF-3, p-IRF-3, p65, p-p65, ERK, p-ERK, Akt, p-Akt, p38, p-p38, STAT2, p-STAT2, and GAPDH at a dilution following the manufacturer’s protocol. After incubation with primary antibodies, horseradish peroxidase-conjugated secondary antibody (CST Biological Reagents) was added to PVDF membranes for 60 min at room temperature (RT). The target proteins were visualized using a western lighting chemiluminescence system (Thermo Fisher Scientific) and quantified by ImageJ software (NIH, Bethesda).

4.11. Statistics. Statistical differences between groups were compared using one-way analysis of variance in GraphPad Prism 8.0 software. Values of p<0.05 were considered as significant.
Disease, Guangzhou Institute of Respiratory Health, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou 510120, China; State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Macau (SAR) 591020, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05264

Author Contributions
R.-F. L., X.-B. Z., and H.-X. Z. made equal contributions to this study.

Notes
The authors declare no competing financial interest.

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