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Short communication

# Drug repurposing of anti-infective clinical drugs: Discovery of two potential anti-cytokine storm agents

Li Su [a,1], Ye Tu [b,1], De-pei Kong [a,1], Da-gui Chen [a], Chen-xi Zhang [a], Wan-nian Zhang [c,d], Chun-lin Zhuang [c,d,*], Zhi-bin Wang [c,*]

[a] Institute of Translational Medicine, Shanghai University, Shanghai, China
[b] Department of Medicine, Shanghai East Hospital, Tongji University, 200120, Shanghai, China
[c] School of Pharmacy, Second Military Medical University, 200433, Shanghai, China
[d] School of Pharmacy, Ningxia Medical University, 750004, Yinchuan, China

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ABSTRACT

Coronavirus Disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus -2 (SARS-CoV-2) has been widely spread in the world with a high mortality. Cytokine storm syndrome (CSS) and acute lung injury caused by SARS-CoV-2 infection severely threaten the patients. With the purpose to find effective and low-toxic drugs to mitigate CSS, entecavir and imipenem were identified to reduce TNF-α using a LPS-induced macrophage model from the anti-infective drug library. Entecavir and imipenem efficiently suppressed the release of inflammatory cytokines by partly intervention of NF-κB activity. The acute lung injury was also alleviated and the survival time was prolonged in mice. In addition, entecavir and imipenem inhibited the release of TNF-α and IL-10 in human peripheral blood mononuclear cells (hPBMCs). Collectively, we proposed that entecavir and imipenem might be candidates for the treatment of CSS.

## 1. Introduction

Coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus -2 (SARS-CoV-2) has been confirmed to infect ~ 17.1 million people in more than 200 countries, territories or areas worldwide, leading to a mortality of 3.9 % (updated on 31 July 2020) [1]. Fever, fatigue, dry cough and pneumonia are the typical clinical symptoms [2–4]. In critical cases, acute respiratory distress syndrome (ARDS), coagulopathy, and septic shock caused by excessive inflammatory response can be fatal [2]. Studies based on biopsy and autopsy indicated that COVID-19 has similar pathological characteristics to SARS or Middle East Respiratory Syndrome (MERS), featured by severe inflammation in lungs, bronchoalveolar damage, and lymphocyte-dominant inflammatory cell infiltration [2,5,6]. In addition to the direct damages by the coronavirus to respiratory epithelial cells and vascular endothelial cells, the excessive inflammatory response usually occurs in disease progression [7,8]. There is growing evidence that a subgroup of patients with severe COVID-19 might have a syndrome with a distinct cytokine storm [9,10]. In SARS-CoV infected patients, an excessive and aberrant host cytokine storm was also reported to be critical factor resulting in an energetic immunopathology and lethal disease [11–13]. The cytokine storm refers to an uncontrolled excessive inflammatory response, which is locally began and further spread in the body through the systemic circulation [14,15]. In the patients infected with SARS-CoV-2, especially moribund patients, the plasma concentrations of inflammation related cytokines have been reported to be significantly increased, including interleukins (IL) -2, -6, -7, and -10, tumor necrosis factor-α (TNF-α), interferon-γ-inducible protein 10 (IP10), granulocyte-colony stimulating factor (G-CSF), monocyte chemoattractant protein -1 (MCP-1), and macrophage

*Abbreviations:* ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CCL-5, chemokine (C-C motif) ligand 5; COVID-19, coronavirus disease 2019; CSS, cytokine storm syndrome; CXCL1, CXC chemokine ligand 1; FDA, U.S. Food and Drug Administration; G-CSF, granulocyte-colony stimulating factor; hPBMC, human peripheral blood mononuclear cell; $IC_{50}$, half-maximal inhibitory concentration; IL, interleukin; IP10, interferon-γ-inducible protein 10; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein -1; MERS, Middle East Respiratory Syndrome; MIP-1α, macrophage inflammatory protein 1 alpha; NLRP3, NLR family PYRIN domain containing-3; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus -2.

* Corresponding authors at: School of Pharmacy, Second Military Medical University, 200433, Shanghai, China.
*E-mail addresses:* zclnathan@163.com (C.-l. Zhuang), methyl@smmu.edu.cn (Z.-b. Wang).
[1] These authors contributed equally.

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inflammatory protein 1 alpha (MIP-1α) [2,16,17]. More pronounced in pathological findings, a large amount of inflammatory immune cell infiltrations in the pulmonary pathology of a COVID-19 patient were found [6,18]. Consequently, severe pulmonary inflammation and cytokine storm are proposed existing in SARS-CoV-2 infection.

The leading cause of mortality is considered to be the respiratory failure from ARDS, and the supportive management of COVID-19 is currently used in clinic [16]. However, there is still no well-accepted effective treatment for COVID-19. The development of novel therapeutics has been mainly focused on antivirals [19,20] (e.g. ribavirin, arbidol, chloroquine phosphate, Kaletra [21], remdesivir [21,22]) and vaccines [9]. However, the cytokine storm is a key node for the patients deteriorating to severe COVID-19 [2,9,16]. Therefore, anti-inflammation has been recognized as a new therapeutic option in clinic, and the treatments include steroids (e.g., prednisone) [21], intravenous immunoglobulin, selective cytokine blockade (e.g., tocilizumab) [9], JAK inhibition (e.g., Baricitinib) [23], Chinese medicines and blood purification [9,21].

Although the cytokine storm of COVID-19 patients is resulted by SARS-CoV-2 infection [9,17], the nosocomial infections of severe patients exacerbate the cytokine storm or lead to the secondary cytokine storm [24]. In clinic, antibacterial therapies (e.g. moxifloxacin, ceftriaxone, azithromycin) have been generally utilized in intensive care unit (ICU) in addition to the antiviral drugs [24]. Given the urgency of the COVID-19 treatments, repurposing of 'old' drugs is an attractive proposition due to the use of de-risked compounds with potentially lower costs and especially shorter development timelines [25]. In the present study, we aim to screen potential anti-inflammatory small molecules from an anti-infective drug library and explore new strategies for directly inhibiting hyperinflammation caused by inflammatory immune cells.

## 2. Material and methods

### 2.1. Materials

Compounds were purchased from TargetMol with a purity of > 98 % (TargetMol). LPS (*E. coli* 0111:B4) was obtained from MilliporeSigma.

### 2.2. Preparation of the peritoneal macrophages

The peritoneal macrophages were obtained from the mice after intraperitoneally (i.p.) injection of 3 mL of 3% thioglycolate as described previously [26]. Briefly, the mice were sacrificed, and the macrophages were isolated by lavage with 5 mL of RPMI 1640 (Gibco Life Technologies), washed twice with PBS after 4 h of adherence, cultured in RPMI at 37 °C and 5% $CO_2$, and finally stimulated with 100 ng/mL LPS to harvest supernatants. The isolated cells were used for cytokine analysis and cell viability assays.

### 2.3. ELISA

TNF-α released by mouse peritoneal macrophages was measured by a Mouse TNF-α ELISA Kit (Invitrogen, BMS607-3TEN) according to the manufacturer's protocol. TNF-α and IL-10 released by hPBMCs were measured by Human TNF-α ELISA Kit (Youda, 1117202) and Human IL-10 ELISA Kit (Youda, 1111002) according to the manufacturer's protocol.

### 2.4. Cell viability assay

Cell Counting Kit-8 (TY0312, Dojindo Molecular Technology, Japan) was used to measure cell viability. Briefly, 10 μL of CCK-8 solution was added, and cells were incubated for 1 h at 37 °C. Absorbance was measured using a Cytation 5 Cell Imaging Multi-mode Reader (BioTek Instruments, USA) at a wavelength of 450 nm.

### 2.5. Anti-inflammatory activity screening

We chose an in vitro model of LPS-stimulated peritoneal macrophages to induce TNF-α secretion, and screened potential anti-inflammatory molecules in the antiviral and antibacterial drug library. Briefly, 100 ng/mL LPS stimulated peritoneal macrophages for 4 h with simultaneous incubation of compounds at a concentration of 10 μM. The cell supernatant was diluted 10-fold and the TNF-α content was measured with a mouse TNF-α Elisa kit obtained from Invitrogen. The remaining cells were subjected to CCK8 assay to detect cytotoxicity. There is one replicate for each compound in the compound library screening experiment. Besides, n = 3 in each group of the vitro experiment to confirm the activity for the candidate compounds.

### 2.6. Animal

Male C57BL/6 J mice (18−22 g) were purchased from the Changzhou Cavens Laboratory Animal Co., China. All mice were kept under an automated 12 h dark-light cycle at a controlled temperature of 22 °C ± 2 °C and a relative humidity of 50%–60% with free access to standard dry diet and tap water. All animal experiments were carried out in adherence with the NIH *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011) and were approved by the Second Military Medical University Committee on Animal Care (EC11-055).

### 2.7. Cytokine storm syndrome (CCS) model

CCS was induced by a single i.p. injection of LPS (15 mg/kg), as described previously [26]. After 4 h, mice were sacrificed and serum was collected. After 8 h, the left bronchus was ligated and 1 mL saline was perfused into right lung lobe to collect bronchoalveolar lavage fluid (BALF), and the left lung was fixed with paraformaldehyde for histological analysis. Serum and BALF were further used for multi-cytokine analysis.

To investigate the effect of drugs on the survival time of CSS model mice, a single i.p. injection of saline (n = 10), entecavir (0.5 and 2 mg/kg, n = 10) and imipenem (25 and 100 mg/kg, n = 10) were performed at 1 h before i.p. injection of a lethal dose of LPS (30 mg/kg) to mice, respectively. After modeling, mice survival was recorded every 2 h until 40 h.

### 2.8. Multi-cytokine measurement

The serum levels of a total of 12 virus-related cytokines were measured by a bead-based immunoassay panel (Mouse Anti-Virus Panel, Cat No: 740622, Biolegend, USA). The BALF levels of a total of 12 inflammatory cytokines were measured by a bead-based immunoassay panel (Mouse Inflammation Panel, Cat No: 740446, Biolegend, USA) on CytoFLEX Flow Cytometer (Beckman Coulter, USA) according to the manufacturer's protocol.

### 2.9. Histological analysis

The left lung lobes of mice were fixed using formalin, and then the tissues were paraffin-embedded. Sections (5 μm) of formalin-fixed tissues were stained with haematoxylin and eosin (H&E) according to the manufacturer's instructions, and were photographed with a microscope (Olympus Corporation, Tokyo, Japan). The histological characteristics of the lung injury (including alveolar edema and hemorrhage, the number of infiltrating leukocytes, and the thickness of the alveolar wall and epithelium) were evaluated. Each histological characteristic was evaluated on a scale of 0–3 (0, normal; 1, mild; 2, moderate; 3, severe).

### 2.10. NF-κB luciferase activity assay

RAW264.7 cells stably transfected with an NF-κB-responsive

luciferase construct, kindly provided by Prof. An Qin (Shanghai Jiaotong University, China), were seeded in 96-well plates at a density of $2 \times 10^5$ cells per well, as previously described [27]. After 24 h, cells were pretreated with entecavir or imipenem at the concentrations of 2.5, 5 and 10 μM for 1 h and stimulated with LPS for 6 h, respectively. Cells were dealt with a luciferase assay system (Promega) and the luciferase activity was calculated using a Cytation 5 Cell Imaging Multi-mode Reader (BioTek Instruments, USA).

### 2.11. Western blotting

Protein samples were separated by 10 % SDS-PAGE, transferred to NC membrane and blocked with 5% non-fat milk in TBST. The membranes were washed with TBST and then incubated with the primary antibody for 6 h at 4 degrees Celsius. The primary antibodies (1:1000) used were all from Cell Signaling Technology, USA and listed as follows: GAPDH antibody(#2118), stat3 antibody(#12640), phospho-stat3 antibody(#98543), SAPK/JNK antibody(#9252), phospho-SAPK/JNK antibody(#4668), p65 antibody(#4764), phospho-p65 antibody (#3033), IKKα antibody(#2682), phospho-IKKα/β antibody(#2697), IκBα antibody(#4812), phospho-IκBα antibody(#2859), p38 MAPK antibody(#8690), phospho-p38 MAPK antibody(#4511), Erk1/2 antibody(#4695) and phospho-Erk1/2 antibody(#4370). Then, the membranes were incubated in HRP-linked goat anti-rabbit IgG Antibody (1:10000, Cell Signaling Technology, USA, #7074) at room temperature for 1 h and signals were detected by chemiluminescence (Bio rad, USA).

### 2.12. Immunofluorescence staining

Isolated peritoneal macrophages in eight-well LabTek slides (PEZGS0816, Millipore, Billerica, Massachusetts, USA) were fixed in 4% paraformaldehyde, blocked with 0.4 % Triton X-100/2% bovine serum albumin at room temperature for 1 h, and then incubated with primary antibodies for p65 (CST, #8242, 1:400 dilution) overnight at 4 °C. After being washed with PBST 3 times, the samples were incubated with Alexa Fluor 488 (Beyotime, A0423, 1:500 dilution) for 1 h and washed again with PBS. Nuclei were stained with DAPI. Images were obtained by confocal microscopy (TCS SP5, Leica, Solms, Germany).

### 2.13. Preparation of the human peripheral blood mononuclear cells (hPBMCs)

hPBMCs were obtained from freshly collected buffy coat fractions from healthy donors at the Tongren Hospital Affiliated to Shanghai Jiaotong University, China. Briefly, hPBMCs were isolated by centrifugation over a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient at 800 g for 20 min at room temperature in a Sorvall RT6000B (DuPont, Wilmington, DE, USA). Most hPBMC isolates were adherence cells that mainly contained macrophages and monocytes. Isolated hPBMCs were cultured in RPMI 1640, 100 U/mL penicillin–streptomycin (Invitrogen Life Technologies), and 10 % heat-inactivated fetal calf serum (Gibco Life Technologies). $3 \times 10^5$ cells were seeded in 96-well plates and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$. hPBMCs were pretreated with entecavir or imipenem at the concentrations of 2.5, 5 and 10 μM at 1 h before LPS (100 ng/mL) stimulation, and the supernatants were harvested at 6 or 12 h after LPS stimulation for cytokine analysis.

### 2.14. Statistical analysis

Data were expressed as means ± SEM. Statistical analyses used Student's *t*-test, two-way ANOVA or Kaplan-Meier Survival Analysis. GraphPad software was used for data analysis. Statistical significance was indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s. not significant.

## 3. Results

### 3.1. Drug screening identifies entecavir and imipenem from an anti-infective drug library as anti-inflammatory agents

In order to identify potential anti-inflammatory agents, an anti-infective drug library containing 251 antiviral and anti-bacterial compounds was screened for their ability to inhibit TNF-α-induced by lipopolysaccharide (LPS) in macrophages. Drugs that inhibit the elevation of TNF-α by 80 % were screened out for further evaluations. Dexamethason acetate was chosen as positive control with 88.4 % TNFα inhibition activity and 83.4 % cell viability (Table S1). As shown in Fig. 1a, the antiviral drug, entecavir, entecavir hydrate, and the antibacterial drug, imipenem exhibited the best inhibitions among the drugs investigated. The antiviral drugs currently used in the treatment of COVID-19 including ribavirin, abidol, ritonavir, remdesivir and lopinavir possessed the TNF-α inhibition of only 1.3~43 % (Fig. 1b). The antibacterial drugs (moxifloxacin, ceftriaxone and azithromycin) recommended in the COVID-19 patients combined with nosocomial infection showed the inhibitions of 38, 29, 41 %, respectively (Fig. 1c). Of note, azithromycin exhibited a significant cytotoxicity (cell viability: 68.6 % at 10 μM). Furthermore, we found that entecavir and imipenem dose-dependently inhibited TNF-α release with the half-maximal inhibitory concentration ($IC_{50}$) as 2.0 and 11 μM, respectively (Fig. 1d, e). Considering that the inhibitory effect of the drugs on TNF-α might be achieved by cytotoxicity, we tested the cytotoxicity of entecavir and imipenem in macrophages by a CCK-8 assay. It was demonstrated that no apparent toxicity was observed in the entecavir-treated macrophages at concentrations lower than 5 μM and slight cytotoxicity to macrophages was shown when the concentration of entecavir exceed 10 μM (Fig. 1f). Imipenem was tolerated in macrophages at the concentrations of up to 40 μM (Fig. 1g).

### 3.2. Entecavir and imipenem inhibit LPS-induced cytokine storm in mice

We used LPS-induced CSS mouse model to evaluate the in vivo inflammatory inhibitory activity. Entecavir or imipenem was administrated at the concentration of 2 or 100 mg/kg at 1 h before LPS injection, and serum was collected at 4 h after LPS injection for further experiment. Twelve cytokines in total were simultaneously measured using a mouse antivirus panel by flow cytometric bead array. Compared with the control group, 9 cytokines including TNF-α, IL family (IL-6, IL-1β, IL-12), chemokines (IP10, MCP-1), interferon family (IFN-α, γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) were significantly decreased by the entecavir treatment (Table 1 and Fig. S1). The other 3 cytokines including IL-10, chemokine (C-C motif) ligand 5 (CCL-5) and CXC chemokine ligand 1 (CXCL1) were slightly downregulated by entecavir without statistical significance. In imipenem treatment group, TNF-α, IL-6, IL-1β, IL-10, IL-12, MCP-1, GM-CSF, IFN-α and IFN-γ were significantly decreased whereas IP10, CCL-5 and CXCL1 showed no apparent change (Table 1 and Fig. S2).

Given that cytokines in BALF could directly represent the inflammation status in the lungs [6,28], we examined cytokines in BALF after 8 h of LPS stimulation. Twelve cytokines in total were simultaneously measured using a mouse inflammation panel by flow cytometric bead array. Compared with the control group, 8 cytokines including TNF-α, IL-6, IL-1α, IL-10, IL-17A, IL-27, MCP-1 and GM-CSF were significantly decreased by the entecavir treatment (Table 2 and Fig. S3). IL-1β, IL-23 and IFN-β, γ showed downregulation without significant difference. In imipenem treatment group, TNF-α, IL-6, IL-1α, IL-1β, IL-10, IL-17A, IL-27, MCP-1 and GM-CSF were significantly decreased whereas IFN-γ and IL-2 were decreased without significant difference (Table 2 and Fig. S4).

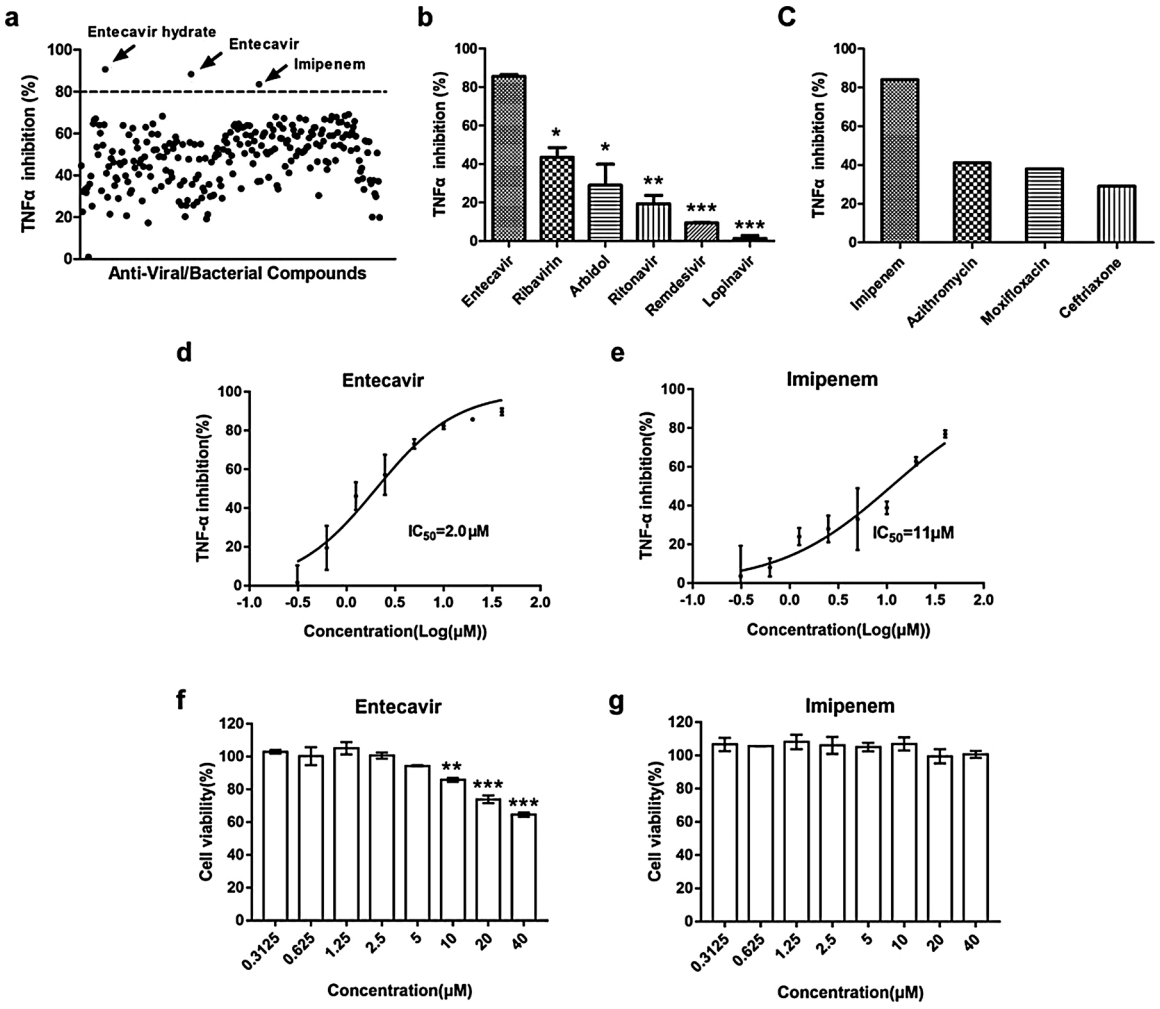


**Fig. 1.** Identification of entecavir and imipenem as anti-inflammatory agents. **a** Entecavir hydrate, entecavir and imipenem suppressed the LPS-induced TNF-α elevation by 80 % in macrophages. **b** Entecavir showed the strongest TNF-α inhibitory effect compared with other antiviral drugs (ribavirin, abidol, ritonavir, remdesivir and lopinavir). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. entecavir. **c** TNF-α inhibitory effect of imipenem compared with moxifloxacin, ceftriaxone and azithromycin. **d** and **e** The dose-response curves for the TNF-α inhibitions of entecavir and imipenem exhibited $IC_{50}$s of 2.0 and 11 μM, respectively. **f** and **g** Cell viabilities of macrophages with entecavir and imipenem treatment at different concentrations. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 0.3125 μM group.

**Table 1**
Clinical feature and experimental results of cytokines tested in mouse serum.

| Cytokines | Elevated in COVID-19 [2, 29] | Higher in Severe Cases [2,29] | Control (pg/mL) | Entecavir (pg/mL) | *P* | Trend | Imipenem (pg/mL) | *P* | Trend |
|---|---|---|---|---|---|---|---|---|---|
| TNF-α | Yes | Yes | 1737.23 ± 69.58 | 1243.4 ± 80.85 | <0.001 | ↓ | 1112.77 ± 35.33 | <0.001 | ↓ |
| IL-6 | Yes | No | 99796.23 ± 2181.37 | 82363.29 ± 5849.85 | 0.002 | ↓ | 77589.85 ± 3969.16 | <0.001 | ↓ |
| IFN-α | No Data | No Data | 648.73 ± 10.58 | 459.80 ± 29.63 | <0.001 | ↓ | 379.79 ± 18.95 | <0.001 | ↓ |
| IFN-γ | Yes | No | 1572.54 ± 96.17 | 871.49 ± 63.01 | <0.001 | ↓ | 861.25 ± 69.08 | <0.01 | ↓ |
| IL-1β | Yes | No | 544.17 ± 32.01 | 355.03 ± 39.37 | <0.001 | ↓ | 270.55 ± 29.68 | <0.001 | ↓ |
| IL-10 | Yes | Yes | 1028.75 ± 46.15 | 889.50 ± 94.24 | 0.24 | – | 878.69 ± 52.15 | 0.052 | – |
| IL-12 | No | No | 243.11 ± 5.65 | 128.27 ± 10.27 | <0.001 | ↓ | 98.62 ± 6.91 | <0.001 | ↓ |
| CXCL1 | No Data | No Data | 30068.23 ± 3912.14 | 26227.69 ± 1877.58 | 0.91 | – | 34548.18 ± 12416.35 | 0.58 | – |
| IP10 | Yes | Yes | 41395.54 ± 1787.44 | 35897.97 ± 1743.83 | 0.007 | ↓ | 37422.71 ± 1594.13 | 0.09 | – |
| MCP-1 | Yes | Yes | 36035.98 ± 578.19 | 28332.26 ± 1719.45 | 0.002 | ↓ | 32098.47 ± 897.84 | 0.023 | ↓ |
| CCL-5 | No Data | No Data | 12449.74 ± 840.43 | 11270.31 ± 570.41 | 0.80 | – | 10020.8 ± 748.60 | 0.25 | – |
| GM-CSF | Yes | No | 223.71 ± 8.98 | 190.58 ± 11.68 | 0.039 | ↓ | 158.05 ± 7.09 | <0.001 | ↓ |

**Table 2**
Clinical feature and experimental results of cytokines tested in mouse BALF.

| Cytokines | Elevated in COVID-19 | Higher in Severe Cases | Control (pg/mL) | Entecavir (pg/mL) | *P* | Trend | Imipenem (pg/mL) | *P* | Trend |
|---|---|---|---|---|---|---|---|---|---|
| TNF-α | No Data | No Data | 26.39 ± 4.53 | 6.24 ± 1.40 | <0.001 | ↓ | 5.86 ± 1.13 | <0.001 | ↓ |
| IL-6 | No Data | No Data | 10176.96 ± 2354.05 | 2542.99 ± 796.29 | 0.006 | ↓ | 1407.38 ± 221.92 | 0.002 | ↓ |
| IFN-β | No Data | No Data | 30.92 ± 5.65 | 17.17 ± 5.28 | <0.001 | – | 13.49 ± 2.86 | 0.02 | ↓ |
| IFN-γ | No Data | No Data | 67.02 ± 20.29 | 27.09 ± 8.45 | <0.001 | – | 32.24 ± 8.20 | 0.39 | – |
| IL-1α | No Data | No Data | 180.92 ± 50.29 | 30.42 ± 9.84 | 0.018 | ↓ | 25.65 ± 2.98 | 0.014 | ↓ |
| IL-1β | No Data | No Data | 28.99 ± 5.99 | 17.67 ± 2.08 | 0.24 | – | 15.98 ± 1.58 | 0.045 | ↓ |
| IL-10 | No Data | No Data | 43.11 ± 12.99 | 9.10 ± 3.56 | <0.001 | ↓ | 2.64 ± 1.60 | 0.008 | ↓ |
| IL-17A | No Data | No Data | 26.81 ± 11.91 | 7.08 ± 2.52 | 0.91 | – | 5.45 ± 1.56 | 0.098 | – |
| IL-23 | No Data | No Data | 9.49 ± 1.87 | 7.73 ± 1.28 | 0.477 | – | 4.82 ± 1.33 | 0.061 | – |
| IL-27 | No Data | No Data | 22.16 ± 3.39 | 6.18 ± 1.52 | <0.001 | ↓ | 2.66 ± 0.58 | <0.001 | ↓ |
| MCP-1 | No Data | No Data | 900.08 ± 285.4 | 207.84 ± 75.47 | 0.80 | – | 195.56 ± 43.45 | 0.035 | ↓ |
| GM-CSF | No Data | No Data | 7.50 ± 1.38 | 3.09 ± 1.09 | 0.031 | ↓ | 3.42 ± 0.75 | 0.02 | ↓ |

### *3.3. Entecavir and imipenem effectively attenuate acute lung injury and prolong the survival in LPS-induced mice*

The histological examinations of two COVID-19 death cases both showed alveolar damage with cellular fibromyxoid exudates, pulmonary edema and interstitial mononuclear inflammatory infiltrates [6,28]. The mice injected with LPS (i.p.) exhibited similar pathological features to ARDS, such as infiltration of inflammatory cells (black arrow), congestion (green arrow) and edema within thickened alveolar (yellow arrow) (Fig. 2a). In contrast, the alveolar structures of mice in both entecavir and imipenem groups were relatively intact, inflammatory cell infiltrations were significantly reduced, and mild alveolar thickening and less bleeding points or congestion were observed (Fig. 2a). Lung injury scores were calculated (Fig. 2b) to show significant protective effects of entecavir (score = 2.6 ± 0.61) and imipenem (score = 2.6 ± 0.54) to the lung tissues compared with that of control group (score = 4.8 ± 0.33). The survival time was prolonged in mice treated with entecavir and imipenem in a dose-dependent manner after an i.p. injection of a lethal dose of LPS (30 mg/kg) compared with that in control mice (Fig. 2c, d).

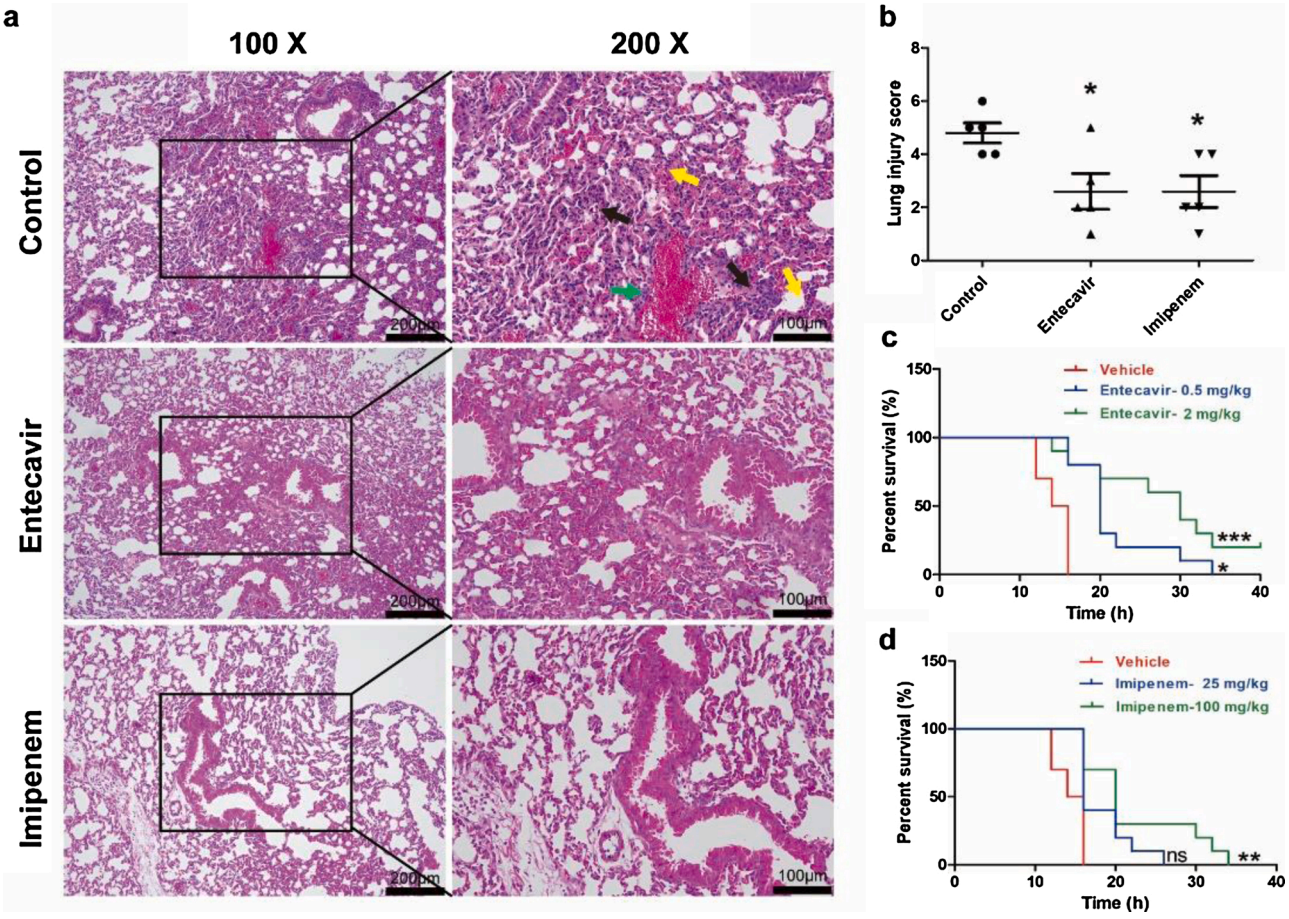


**Fig. 2.** Entecavir and imipenem effectively attenuated acute lung injury and prolong the survival in LPS-induced mice. **a** Representative images of lung H&E staining of control, entecavir and imipenem treatment groups. Black, green and yellow arrows indicated infiltration of inflammatory cells, congestion and edema within thickened alveolar, respectively. **b** Lung injury scores of control, entecavir and imipenem treatment groups (n = 5). Scale bars, 100 or 200 μm as indicated. **c** and **d** Survival time of LPS-induced CSS mice in control, entecavir (0.5, 2 mg/kg) and imipenem (25, 100 mg/kg) groups (n = 10). Kaplan–Meier analysis was performed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, no significance.

### 3.4. Entecavir and imipenem decrease NF-κB activity in LPS-induced macrophage

NF-κB is an important transcriptional regulator in cells that participated in inflammatory responses, of which the activation induces the expression of multiple genes and production of cytokines consequently leading to cytokine storm [30]. Entecavir and imipenem dose-dependently inhibited LPS-induced NF-κB transcriptional activity in the RAW264.7 cells with a NF-κB reporter luciferase system (Fig. 3a, b). The activation of NF-κB pathway (phosphorylation of IκBα, IKKα/β, and p65) was significantly inhibited after entecavir intervention (Fig. 3c), while the phosphorylation of p65 and degradation of IκBα were also decreased after imipenem treatment (Fig. 3d). Furthermore, entecavir and imipenem could significantly inhibit LPS-stimulated p65 nuclear translocation (Fig. 3e). However, the activation of MAPK and STAT pathway were not obviously affected (Fig. 3c, d).

### 3.5. Entecavir and imipenem inhibit multi-cytokine releases in LPS-induced hPBMCs

Considering the translational value of entecavir and imipenem in clinical practices, the releases of a pro-inflammatory cytokine TNF-α and an anti-inflammatory cytokine IL-10 were tested in LPS-induced hPBMCs, which were obtained from two healthy donors (Figs. 4 and S5). Entecavir (Fig. 4a, b) and imipenem (Fig. 4c, d) both significantly counteracted the levels of TNF-α and IL-10 at 6 h and 12 h in a time- and dose-dependent manner without apparent toxicity.

## 4. Discussion

CSS caused by SARS-CoV-2 infection plays a critical role in inducing ARDS [17]. Repurposing marketed drugs to target CSS and reduce immunopathology could be an alternative strategy for clinical therapy [9,31]. Recently, a clinical trial using tocilizumab to block the IL-6 receptor and inflammatory signals has been launched (ChiCTR2000029765). In addition to the proposed JAK inhibitor Baricitinib [23], there are still no small molecule reported to possess anti-inflammatory effect. In the absence of specific drugs of COVID-19, current antiviral and antibacterial therapies have been widely attempted to combat pathogenic microorganism [20,22]. Direct inhibition of massive cytokines released by hyperactivated immune cells using the existing anti-infective drugs (e.g. antiviral and antibacterial) may achieve additional therapeutic effects to save severe COVID-19 patients [23].

Viruses and bacteria induce immune cell activation and release of cytokines are dependent on Toll-like receptors (TLRs) [32]. SARS-CoV has been reported to induce considerable levels of pro-inflammatory cytokines via the TLR7 and TLR8 [33], which is different from the model of bacteria to recognize TLR4. However, TLR4 could also be upregulated by SARS-CoV infection [34], which leads to increased binding of LPS and enhanced inflammatory reaction [35]. Although they recognize different subtypes of TLRs, the induction of inflammatory cytokines depends on the activation of NF-κB. Moreover, SARS-CoV nucleocapsid protein can significantly activate NF-κB [36]. We assumed that SARS-CoV-2 may share a similar mechanism on cytokine storm. LPS activates immune cells such as monocytes and macrophages, causing the synthesis and release of inflammatory cytokines [37]. Considering that the goal is to find drugs that directly inhibit the release of cytokines, we stimulated macrophages with LPS to quickly screen the target compounds, and the candidate compounds entecavir and imipenem showed, to some extent, inhibition of NF-κB activation. TNF-α is one of the central cytokines involved in inflammation initiation and amplification in virus infections [38], and is reported to be elevated in critical COVID-19 cases [2], suggesting it as a proper indicator for in vitro drug screening.

A recent study reported that viral infections caused sepsis in 100 %

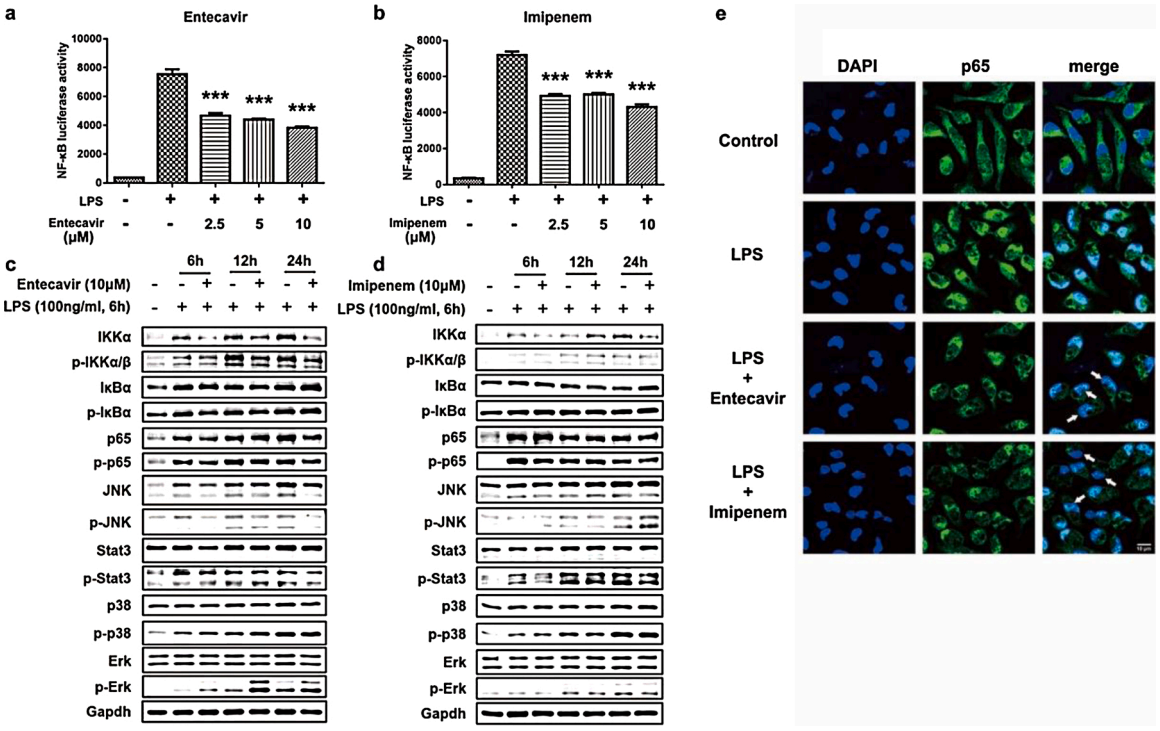


**Fig. 3.** Entecavir and imipenem effectively inhibited NF-κB pathway activity in LPS-stimulated macrophage. **a** and **b** RAW264.7 cells were co-cultured with entecavir and imipenem at concentrations of 2.5, 5 and 10 μM at 1 h before LPS stimulation. The activity of NF-κB luciferase was upregulated in all groups after 8 h, and there was a significant decline in cells co-cultured with entecavir and imipenem in a dose-dependent manner. **c** and **d** The activation of the NF-κB, MAPK and STAT pathway in LPS-stimulated macrophages after the treatment of entecavir and imipenem. **e** p65 nuclear translocation in LPS-stimulated macrophages after the treatment of entecavir and imipenem. Scale bars, 10 μm as indicated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

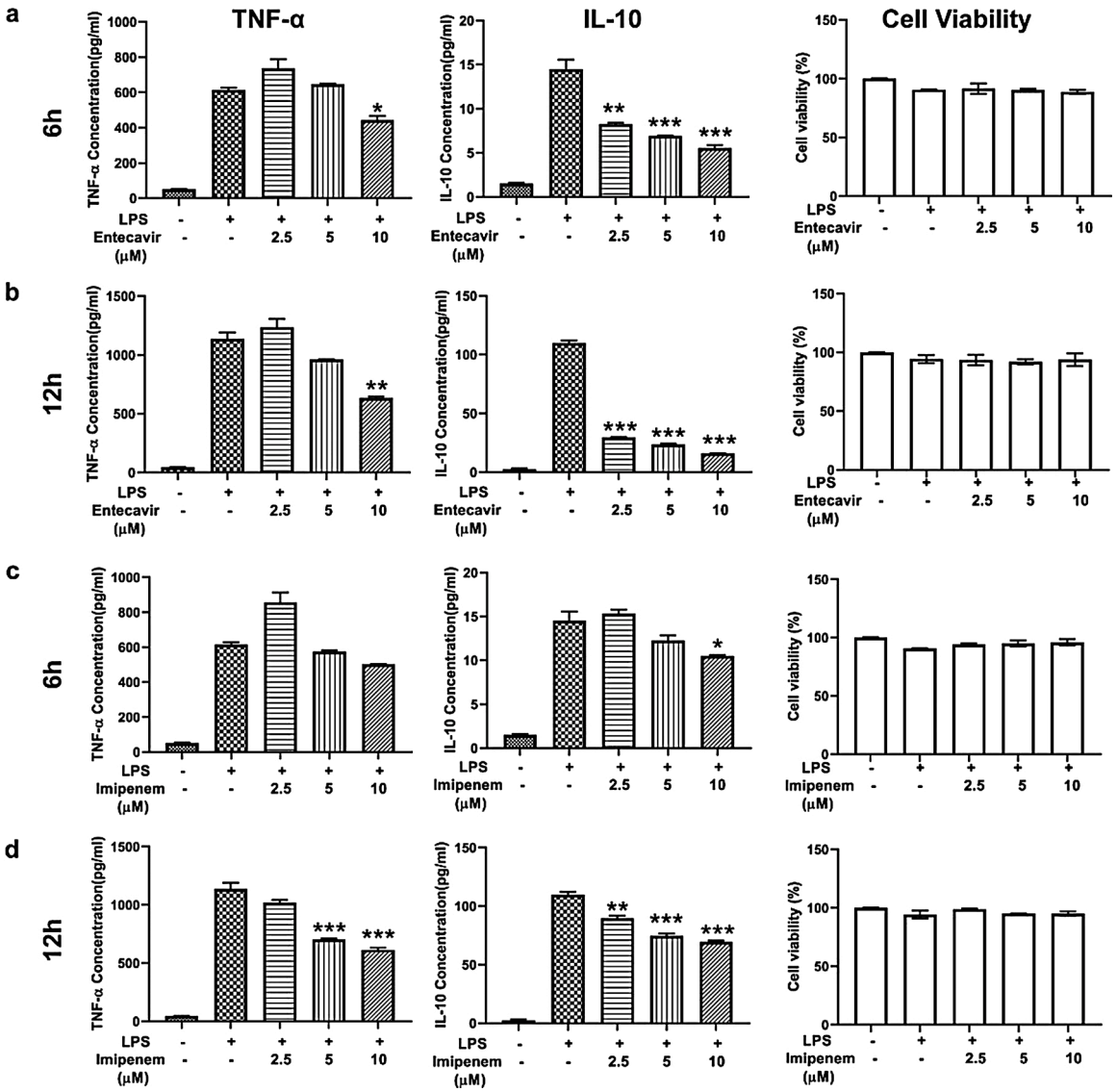


**Fig. 4.** Entecavir and imipenem inhibite cytokine release in LPS-induced hPBMCs from a health donor. TNF-α and IL-10 concentrations were elevated by LPS stimulation. **a** and **b** Entecavir reduced TNF-α and IL-10 release in a time (6 and 12 h)- and dose (2.5, 5, 10 μM)-dependent manner. **c** and **d** Imipenem reduced TNF-α and IL-10 release in a time (6 and 12 h)- and dose (2.5, 5, 10 μM)-dependent manner. Entecavir and imipenem showed no toxicity toward hPBMCs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. LPS model group.

death cases, 42 % survival cases of the COVID-19 patients [39]. Cytokines induced by bacteria infection [40] were similar to those present in COVID-19 patients [2]. In LPS-induced CSS model, we simultaneously investigated cytokines related with antivirus and inflammation covering clinically relevant cytokines in COVID-19. Entecavir was approved in 2005 by the U.S. Food and Drug Administration (FDA) for the treatment of hepatitis B virus (HBV) infection by targeting RNA-dependent RNA polymerase (RdRp) [41,42]. Dr. Qiang Qu from Xiangya Hosptial, Central South University, China, predicted that the entecavir triphosphate could well bind with the RdRp model of SARS-CoV-2 by computational homology modeling [43]. Entecavir has also been used for prevention of COVID-19 in some high-risk groups and suspected population, and other viral respiratory infection [43]. Interestingly, a RdRp, EV71 3D protein, was found to stimulate the activation of NLR family PYRIN domain containing-3 (NLRP3) inflammasome, and the release of IL-1β through direct binding to NLRP3 [44]. In our study, we found that entecavir could significantly inhibit the secretion of IL-1β and other cytokines such as IL-6 and TNF-α, etc.

Occurrences of secondary infections were reported to be observed in 50 % death cases of severe COVID-19 patients [39]. In a clinical characteristics of 138 hospitalized patients with COVID, -19many received antibacterial therapies (moxifloxacin, 89 [64.4 %]; ceftriaxone, 34 [24.6 %]; azithromycin, 25 [18.1 %]) [24]. However, these three broad-spectrum antibiotics did not exhibit apparent anti-inflammatory activity (Fig. 1c). Imipenem, a β-lactam broad-spectrum antibiotics wildly used in clinical practice for more than 30 years, could significantly reduce inflammatory cytokines. Hence, imipenem may be a candidate for COVID-19 patients combined with nosocomial bacterial infections.

## 5. Conclusion

In summary, entecavir and imipenem were identified to effectively prevent the development of CSS in the serum and BALF of LPS-induced mice and isolated hPMBCs. They can also effectively alleviate the lung injury and prolong the survival time of LPS-induced mice. Emerging evidences have supported that damage of lung and other organs swept by CSS may be a predominated cause of death in severe COVID-19 patients [45]. It is suggested that retrospective studies may be performed to further determine their anti-CSS effects in COVID-19 patients

combined with hepatitis or bacterial infection.

## Author contributions

L. S., C.-L. Z., W.-N. Z, Z.-B. W. conceived and designed the experiments; L. S., Y. T., D.-P. K., D.-G. C., C.-X. Z., Z.-B. W. participated in the experiments; L. S., Y. T., Z.-B. W. analyzed the data; L. S., C.-L. Z., Z.-B. W. wrote the manuscript; all the authors provided the final approval of the manuscript.

## 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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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110643.

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