

Indomethacin has a potent antiviral activity against SARS CoV-2 in vitro and canine coronavirus in vivo

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Abstract:

Background: The outbreak of SARS CoV-2 has caused ever-increasing attention and public panic all over the world. Currently, there is no specific treatment against the SARS CoV-2. Therefore, identifying effective antiviral agents to combat the disease is urgently needed. Previous studies found that indomethacin has the ability to inhibit the replication of several unrelated DNA and RNA viruses, including SARS-CoV.

Methods: SARS CoV-2 pseudovirus-infected African green monkey kidney VERO E6 cells treated with different concentrations of indomethacin or aspirin at 48 hours post infection (p.i). The level of cell infection was determined by luciferase activity. Anti-coronavirus efficacy in vivo was confirmed by evaluating the time of recovery in canine coronavirus (CCV) infected dogs treated orally with 1mg/kg body weight indomethacin.

Results: We found that indomethacin has a directly and potently antiviral activity against the SARS CoV-2 pseudovirus (reduce relative light unit to zero). In CCV-infected dogs, recovery occurred significantly sooner with symptomatic treatment + oral indomethacin (1 mg/kg body weight) daily treatments than with symptomatic treatment + ribavirin (10-15 mg/kg body weight) daily treatments ($P = 0.0031$), but was not significantly different from that with symptomatic treatment + anti-canine coronavirus serum + canine hemoglobin + canine blood immunoglobulin + interferon treatments ($P = 0.7784$).

Conclusion: The results identify indomethacin as a potent inhibitor of SARS CoV-2.

Keywords: 2019-nCoV; COVID-19; SARS CoV-2; Indomethacin

Introduction: Since 12 December 2019, there has been an outbreak of Acute Respiratory Syndrome caused by a new coronavirus (SARS CoV-2). The disease caused by the virus

was named Coronavirus Disease 19 (COVID-19). As of March 20, 2020, SARS CoV-2 has been reported in 76 countries and 308,613 cases and 13,844 deaths have been confirmed, with an estimated mortality risk of 4%. Common symptoms at onset of illness were fever, cough, myalgia and fatigue; fewer common symptoms were sputum production, headache, haemoptysis, and diarrhea. Complications included acute respiratory distress syndrome, acute cardiac injury¹.

It is currently believed that SARS CoV-2 may be transmitted from bats to humans². Angiotensin-converting enzyme 2 (ACE2) is the main host cell receptor of SARS CoV-2 and plays a crucial role in the entry of virus into the cell to cause infection³⁻⁵. High ACE2 expression was identified in type II alveolar cells (AT2) of lung, esophagus upper and stratified epithelial cells, absorptive enterocytes from ileum and colon, and bladder urothelial cells⁶⁻⁸.

New interventions are likely to require years to develop. Given the urgency of the SARS CoV-2 outbreak, we focus here on the potential to repurpose existing drugs approved for treating infections caused by RNA virus, based on therapeutic experience with two other infections caused by human coronaviruses: severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS)⁹. Cyclooxygenases (COXs) play a significant role in many different viral infections with respect to replication and pathogenesis¹⁰. Cyclopentone COX inhibitor indomethacin has been widely used in the clinic for its potent anti-inflammatory and analgesic properties¹¹. In addition to the anti-inflammatory/analgesic action, indomethacin has been known to also possess antiviral properties, including canine coronavirus (CCV) and SARS-CoV¹²⁻¹⁴.

CCV has certain sequence homology with SARS CoV-2¹². CCV-infection in dogs are common in winter and spread rapidly. Puppies have higher morbidity and mortality than adult dogs¹⁵. The main symptoms of dogs with CCV-infection are those of the digestive system, such as vomiting and diarrhea, and sometimes respiratory symptoms¹⁶.

In the present report we described how indomethacin was found to potently inhibit SARS CoV-2 *in vitro*, and efficiently treat CCV-infected dogs.

Methods:

Cell culture and treatment

VERO E6 cells were grown at 37°C in a 5% CO₂ humidified atmosphere in Modified Eagle Medium (MEM), supplemented with 10% fetal calf serum (FCS). Indomethacin was dissolved in absolute ethanol at a concentration of (0, 0.1, 1, 5, 10, 50, 100, 500 µM) and diluted in culture medium immediately before use. Unless otherwise specified, indomethacin and Aspirin were added immediately after the 1 h adsorption period and maintained in the medium for the duration of the experiment. Controls received equal amounts of appropriate diluent Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to MTT formazan

conversion assay.

Coronavirus infection and titration

The cDNA of the SARS CoV-2 (SARS CoV-2 S , GenBank: MN908947.3). The SARS CoV-2 pseudotyped virus HIV/SARS was produced by a similar method which was described previously¹⁷. Briefly, 10 µg pNL4-3-Luc-R-E-pro¹⁸ and 10 µg pTSh were co-transfected into VERO E6 cells in 10 cm dishes. Supernatants were harvested 48 h later and used in infection assays. The pseudotyped virus was purified by ultracentrifugation through a 20% sucrose cushion at 50,000 g for 90 min, resuspended in 100 µl PBS. The supernatant containing 5 ng pseudotyped virus (p24) was used to infect cells in 24-well plates (4–8×10⁴ cells/well), and treated with different concentrations (0, 0.1, 1, 5, 10, 50, 100, 500 µM) of indomethacin or Aspirin after the 1 h adsorption period. The cells were lysed at 48 h post infection (p.i). Twenty microliters of lysate was tested for luciferase activity by the addition of 50 µl of luciferase substrate and measured for 10 s in a Wallac Multilabel 1450 Counter. After incubation at 37°C for 30 min, 100 µl mixtures were added to VERO E6 cells in 96-well plates. The cells were lysed at 48 h p.i and tested for luciferase activity as described above¹⁹.

Animal maintenance

The enrolled dogs were confirmed for diagnosis of CCV infection with a Canine Coronavirus test kit and between 2-3 days of the onset of symptoms. We enrolled 26 dogs which were 3-6 months old and medium-sized. There were several types of these dogs, including poodles and Pekingese dogs. Dogs were grouped into three populations based on the treatment scheme as follows: (1) symptomatic treatment + ribavirin (10-15 mg/kg body weight) daily, 8 dogs; (2) symptomatic treatment + anti-canine coronavirus serum + canine hemoglobin + canine blood immunoglobulin + interferon, 9 dogs; (3) symptomatic treatment + oral indomethacin (1 mg/kg body weight) daily²⁰, 9 dogs. The time of recovery was determined by the disappearance of symptoms and a negative diagnosis by the canine coronavirus test kit. Humane standards were adhered to for all assessments. The study procedure was approved by Animal Ethics Committee of United Animal Hospital.

Statistical analysis

In virus titration experiments, statistical analysis was performed using Student's t-test for unpaired data. Data were expressed as the mean ± SD and P-values of <0.05 were considered significant.

Results:

1. Indomethacin is a potent inhibitor of SARS CoV-2 *in vitro* replication

To investigate the effect of indomethacin on SARS CoV-2 replication, African green monkey kidney VERO E6 cells were infected with SARS CoV-2 and treated with different concentrations (0, 0.1, 1, 5, 10, 50, 100, 500 μ M) of indomethacin or Aspirin after the 1 h adsorption period. The level of cell infection was determined by luciferase activity at 48 h p.i. Surprisingly, indomethacin was found to possess a remarkable antiviral activity, reducing viral particle production dose-dependently with an IC₅₀ (inhibitory concentration 50%) of 1 μ M, and selective index of 500, and caused a dramatic reduction relative light unit to zero at 48 h p.i. in VERO E6 cells (Figure 1A). Oral administration of 50 mg of indomethacin generates a peak plasma concentration of the drug ranging from 7 μ M to 11 μ M, a concentration which is 10 times of the drug's IC₅₀²¹. These concentrations of indomethacin did not affect VERO E6 cell proliferation (Figure 1B) indicating that even at 500 μ M indomethacin still does not show any cytotoxicity to VERO E6 cell.

2. Antiviral activity of indomethacin in CCV-infected dogs

In order to evaluate whether indomethacin could also be effective against CCV *in vivo*, we analyzed the rate of recovery in dogs with treatment schemes as follows: (1) symptomatic treatment + ribavirin (10-15 mg/kg body weight) daily; (2) symptomatic treatment + anti-canine coronavirus serum + canine hemoglobin + canine blood immunoglobulin + interferon; (3) symptomatic treatment + oral indomethacin (1 mg/kg body weight) daily. The results showed that recovery occurred significantly sooner with the (3) treatments than with the (1) treatments ($P = 0.0031$, Fig.2A), but was not significantly different from that with the (2) treatments ($P = 0.7784$, Fig.2B). Moreover, in the group of with the (1) treatment scheme were 8 dogs, and 3 died (3/8); with the (2) scheme were 9 dogs, and 1 died (1/9); with the (3) scheme were all survived from this disease (9 dogs).

Discussion: Given the urgency of the SARS CoV-2 outbreak, we focus here on the potential to repurpose existing agents approved or in development for treating infections caused by HIV, hepatitis B virus (HBV), hepatitis C virus (HCV) and influenza^{9,22}. Indomethacin has been known to possess antiviral properties¹²⁻¹⁴, and used for a long time as a potent anti-inflammatory drug, acting by blocking the activity of cyclooxygenase-1 and -2 (COXs), activity and inhibiting pro-inflammatory prostaglandin synthesis²³. Indomethacin reduces the synthesis of prostaglandin (PG) by inhibiting cyclooxygenase, and inhibiting the formation of painful nerve impulses in inflammatory tissues thus suppressing the inflammatory response¹⁰.

In our experiments, we tested the COX inhibitors indomethacin and aspirin. Our results show that indomethacin has potent, direct antiviral activity against the SARS CoV-2 (reduce the relative light unit to zero) in VERO E6 cells (Figure 1A), and does not show cytotoxicity to VERO E6 cells (Figure 1B). The current study, and reported by others, suggest that indomethacin has strong anti-Coronavirus activity *in vitro*^{12,13}, including SARS COV-2, while aspirin does not. This suggests that indomethacin's antiviral activity is not achieved by some mechanisms other than COX inhibition.

In our study of CCV-infected dogs, the results show that in terms of anti-CCV efficacy, indomethacin can achieve a similar efficacy as treatment with anti-canine coronavirus serum, canine hemoglobin, canine blood immunoglobulin and interferon treatments effect ($P = 0.7784$, Fig.2B), and superior efficacy than the treatment with ribavirin ($P = 0.0031$, Fig.2A). These findings were consistent with other report and revealed that indomethacin also shows significant anti-CCV *in vivo*¹². Its mechanism, however, remains largely unknown.

Although the underlying mechanism requires further investigation, the results of indomethacin anti-SARS-CoV show that indomethacin does not affect virus infectivity, binding or entry into target cells, but acts early on the coronavirus replication cycle, selectively blocking viral RNA synthesis¹². The only clear indomethacin antiviral mechanism is that, instead, triggers a cellular antiviral defense mechanism by rapidly and effectively activating PKR in an interferon- and dsRNA-independent manner¹³. PKR plays a critical role in the antiviral defense mechanism of the host, acting as a sensor of virus replication and²⁴, upon activation, leading to eIF2 α phosphorylation and block of protein synthesis in virally infected cells²⁵.

To investigate possible antiviral mechanisms not related to COX inhibition, we utilized Plex Research's AI search engine platform²⁶. A Plex analysis of indomethacin identified additional targets that are directly modulated by indomethacin or its close analogs: aldo-keto reductase (AKR1C3, AKR1C4, AKR1C2)²⁷⁻²⁹, aldose reductase (AKR1B1)³⁰, peroxisome proliferator activated receptor gamma (PPARG)³¹⁻³³, and cannabinoid receptor 2 (CB2)³⁴. Of these, only AKR1B1 and PPARG are expressed in VERO cells³⁵.

There are few reports about the role of aldo-keto reductases in inflammation. Aldo-keto reductase was found to reduce peroxidation derived lipid aldehydes, such as 4-hydroxytrans-2-nonenal and their glutathione conjugates, to corresponding alcohols, 1,4-dihydroxy-nonene and glutathionyl-1,4-dihydroxynonene, respectively, which take part in the inflammatory signaling³⁶. Inhibition of aldose reductase was found to significantly prevent the transfer of the inflammatory signals induced by multiple factors including allergens, cytokines, growth factors, endotoxins, high glucose, and autoimmune reactions at the cellular level as well as in animal models³⁷.

It has been demonstrated that PPARG agonists inhibit the production of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6³⁸. Macrophages loss of PPARG leads to altered differentiation kinetics³⁹. PPARG has been described to be important for resolving inflammation and maintaining homeostasis.

Cannabinoids receptor 2 (CB2), which influences the immune system, viral pathogenesis, and viral replication. The anti-inflammatory and immunoregulatory action of cannabinoids are CB2 dependent⁴⁰. Activated CB2 receptors play a significant role in leukocyte and endothelial migration, activation, and interaction, which is related to the anti-inflammatory and immunomodulatory effects of CB2⁴¹. CB2 receptors suppress

chemokine induced chemotaxis of neutrophils, lymphocytes, macrophages, monocytes, and microglia by inhibiting leukocyte migration mediated by RhoA activation^{42,43}. In addition, CB2 can also inhibit the recruitment of neutrophils⁴⁴. Therefore, CB2 plays a crucial role in maintaining immune homeostasis and controlling the magnitude of the immune response through negative regulation.

IL-6 is a soluble mediator with a pleiotropic effect on inflammation and immune response⁴⁵. It is generally known as endogenous pyrogens which can induce fever. It was shown that an increase in intrahypothalamic concentrations of IL-6 and TNF-like activity during LPS-induces fever⁴⁶. A recent report indicates that indomethacin can reduce the cytokine-mediated IL-6 release by reducing LPS⁴⁷. COVID-19 patients show a "cytokine storm" phenomenon, while IL-6 in the body is significantly increased^{47,48}. The latest treatment guidelines for COVID-19 include the reduction of concentration of IL-6.

During the preparation of this manuscript, a preliminary report emerged describing a proteomic analysis of interactions between SARS CoV-2 viral proteins and 332 human proteins⁴⁹. In this report, the viral NSP7 protein was found to interact with human PTGES2. PTGES2 is expressed in VERO cells⁵⁰ and is inhibited by indomethacin ($IC_{50} = 750 \text{ nM}$)⁵¹. It is also inhibited by aspirin, however much less potently ($IC_{50} = 35 \mu\text{M}$)⁵¹. NSP7, together with NSP8, forms the viral primase complex, which is part of the viral RNA polymerase machinery^{52,53}. Inhibition of NSP7/NSP8 complex function through inhibition of PTGES2 would be consistent with the selective blockage of viral RNA synthesis observed after indomethacin treatment of SARS-CoV¹².

Although the mechanisms underlying the antiviral activity of indomethacin against coronavirus infection require further investigation, our results herein show that indomethacin inhibits SARS CoV-2 both *in vitro* and *in vivo*.

Previously, we have used nebulized indomethacin to treat patients with bronchorrhea or excessive phlegm caused by bronchioloalveolar cell carcinoma or COPD and found that indomethacin can dramatically reduce the bronchorrhea sputum and abate dyspnea. Due to patients with COVID-19 often exhibit excessive phlegm which may causes severe dyspnea, indomethacin not only directly inhibit virus as our current study identified, but it also may reduce sputum secretion and relieve symptoms.

NSAIDs are commonly used as antipyretics and/or analgesics in treating Community-acquired pneumonia. In some survey or observational study, up to half of the patients diagnosed with CAP were treated with NSAIDs⁵⁴⁻⁵⁶. Among these NSAIDs, indomethacin is used less frequently than other paracetamol, fenbufen, ibuprofen or aspirin, mainly due to the relatively frequent gastrointestinal side effects of oral indomethacin, but indomethacin rarely causes serious side effects. Given the fact that indomethacin has been widely used in the clinic for several decades and its safety profile is well defined⁵⁷, we suggest that indomethacin should be assessed further in a prospective clinical study as a treatment of COVID-19 patients.

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Competing Interests

Tianhong Xu and Xuejuan Gao are founder and employee of BaylorOracle Inc. a biotechnology company that develops countermeasures to emerging viruses. Tianhong is listed as an inventor on two patent applications describing a new formulation of indomethacin and its method of use.

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Figure legend:

Figure 1. Indomethacin is a potent inhibitor of SARS CoV-2 in vitro replication. (A). VERO E6 cells were infected with SARS CoV-2 and treated with different concentrations of the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin (INDO, red line) (0, 0.1, 1, 5, 10, 50, 100, 500 μ M) or Aspirin (black line) (0, 0.1, 1, 5, 10, 50, 100, 500 μ M) after the 1 h adsorption period. The level of cell infection was determined by luciferase activity in the supernatant of infected cells at 48 h p.i. (B). Cell viability (black line) determined by MTT assay in uninfected indomethacin-treated cells is expressed as percentage of MTT conversion in untreated control, and (red line) indicates relative light unit treated with as (A). Data expressed in relative light unit represent the mean \pm SD of triplicate samples from a representative experiment of three with similar results. P <0.05 indicates a significant difference.

Figure 2. Antiviral activity of indomethacin in CCV-infected dogs. This figure shows the rate of recovery in CCV-infected dogs with the treatment schemes as follows: (1) symptomatic treatment + ribavirin (10-15 mg/kg body weight) daily; (2) symptomatic treatment + anti-canine coronavirus serum + canine hemoglobin + canine blood immunoglobulin + interferon; (3) symptomatic treatment + oral indomethacin (1 mg/kg body weight) daily. The time of recovery was determined by the symptoms disappeared and the diagnosis of CCV test paper turned negative. Both in (A) and (B) red line indicates treatment with the (3) scheme. Blue line indicates treatment with the (1) scheme in (A), however indicate treatment with the (2) scheme in (B). P <0.05 indicates a significant difference.

Figure 1.

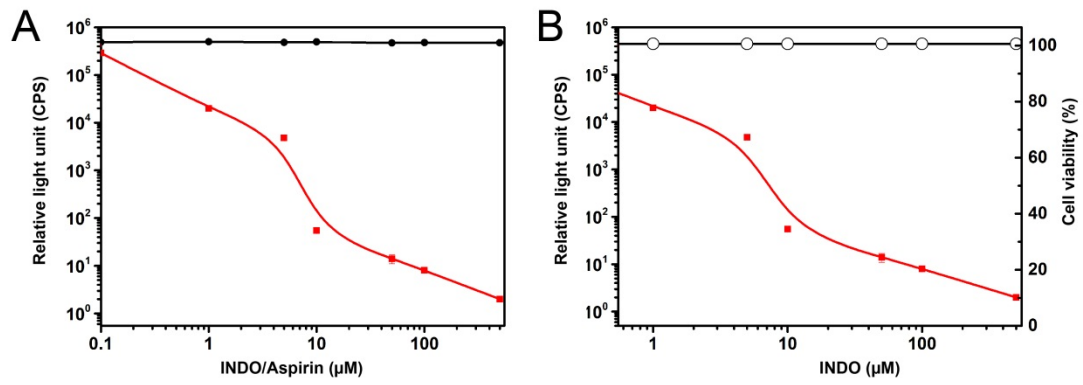


Figure 2.

