Dabigatran and Remdesivir Synergistically Inhibit Coronavirus Replication

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Research Article

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Abstract

Background & Aims: Coronavirus-19 (COVID-19) due to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV-2) is an ongoing global pandemic causing more than three million deaths. Protease inhibitors had been shown to decrease viral entry. However, the role of dabigatran, an inhibitor of multiple proteases, on coronavirus remains unknown.

Methods: MRC-5 cells, HCT-8, or Huh-7 cells were infected with Beta-coronavirus OC43 and SARS-CoV-2. Cytopathic effects (CPE) were monitored by imaging. Viral load was measured by quantitative RT-PCR. Viral protein was detected by Western blot.

Results: Camostat, a serine protease inhibitor, had no effect on the replication of OC43 and SARS CoV-2 even at higher doses. Dabigatran inhibited replication, viral entry and CPE of OC43 in a dose-dependent manner. Dabigatran and Remdisivir synergistically inhibited OC43 virus replication.

Conclusions: Dabigatran may be beneficial in treating SARS-CoV-2 both for anticoagulation and viral replication inhibition need to be evaluated further.

Introduction

Since the first case in December 2019 at Wuhan, China, Coronavirus-19 (COVID-19) has continued as an ongoing global pandemic with 165,069,258 confirmed cases worldwide and about 3,422,907 deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019. 5/21/2021). COVID19 is caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus), a family of beta Coronaviruses that also includes the MERS-CoV (Middle East Respiratory Syndrome) and SARS CoV (Severe Acute Respiratory Syndrome coronavirus). [1–5]

This group of viruses is highly contagious and pathogenic. They share a specific strategy for entry and survival in the infected cell.[6] The maturation of coronaviruses involves a cascade of proteolytic events on the viral polyproteins to control viral gene expression and replication. [7]Host cell proteases also play a critical role in coronavirus tropism and pathogenesis.[8] For example, cleavage of spike (S) glycoprotein on SARS-CoV-2 surface by transmembrane protease/serine subfamily member 2(TMPRSS2) is essential for viral entry into cells. [9–11] Therefore, pharmacologic agents inhibiting the TMPRSS2 have been suggested to treat COVID19. Camostat mesylate, a serine protease inhibitor, which has been approved in Japan for treatment of pancreatitis, has been shown to partially block SARS-CoV cell entry [12] and significantly reduce mortality following SARS-CoV infection in animal studies. [13] Recent studies also showed camostat effectively blocked SARS-CoV-2 entry into lung cells. [11] Clinical trials are ongoing to assess the impact of camostat on the course of COVID-19 (NCT04321096, registered on March 25, 2020; NCT04353284, registered on April 20, 2020). Additionally, many other host proteases are involved in viral invasion, viral cell cycle, and host immunity. Targeting proteases remains a promising treatment for COVID-19. Many protease inhibitors have been approved by FDA (Food and Drug Administration) for a

variety of indications. Among them, dabigatran is a serine protease inhibitor that effectively inhibits the activity of thrombin, trypsin, and multiple other proteases. In addition, it has been approved by FDA as an anticoagulant that can be used to treat coagulopathy commonly seen in COVID-19 patients. However, its effects on treating COVID-19 have not been studied yet. In this study, we aimed to investigate the utility of dabigatran on SARS-CoV-2.

Results

Camostat does not inhibit OC43 replication

Due to safety concerns, readily available Biological Safety Level 2 coronaviruses, OC43 was used in our study. OC43 belongs to the same beta-coronavirus family, like SARS-CoV-2, and shares similar cell entry and viral replication machinery. It has been shown that proteases are important for multiple steps of the coronavirus cycle including cell entry, the maturation of the polyprotein, and the assembly of the secreted virions for further diffusion. Camostat is a protease inhibitor that has been shown to partially block SARS-CoV and SARS-CoV2 cell entry [12–14]. We decided to evaluate its effect on viral replication.

MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without camostat (100mM). The supernatant was collected 48h later and subjected to q-RT-PCR for viral load quantification. Quantitative RT-PCR results indicated that, compared with control, camostat did not change the Ct significantly suggesting camostat has minimal effect on OC43 replication (Fig. 1A). Western blot showing camostat did not affect viral protein expression in infected cells (Fig. 1B) further confirmed that camostat had no effect on OC43 viral replication. Similar results were seen in HCT-8 cells and Huh-7 cells (data not shown).

Camostat does not improve CPE of OC43

The cytopathic effect (CPE) of camostat was assessed using the OC43 virus in MRC-5 cells. One hour after the OC43 virus was added to the MRC-5 cell culture, camostat (25, 50, 100μM) was added to the culture medium. On day 3, cells treated with camostat showed similar CPE compare to control cells (Fig. 1C). Similar results were seen in HCT-8 cells and Huh-7 cells (data not shown).

Camostat does not prevent OC43 entry

We next evaluated the OC43 replication in MRC-5 cells pretreated with camostat, which has been shown to partially blocked SARS-CoV and SARS-CoV2 cell entry. [12–14] OC43 viruses were inoculated onto MRC-5 cells that had been pre-treated with camostat (100mM) for 1 hour. Cell supernatants were collected and RNA was extracted. Real-time RT-PCR showed camostat did not change the replication curve of OC43 in MRC-5 cells, suggesting no major inhibition of OC43 cellular entry (Fig. 2A). Similarly, Western blot of the viral protein showed no change of viral protein in either camostat pre-treated (camostat before viral inoculation) or post-treated (camostat treatment after viral inoculation) samples compared to control (Fig. 2B). Similar results were seen in HCT-8 cells and Huh-7 cells (data not shown).

Dabigatran dose-dependently inhibited OC43 replication

Dabigatran is a protease inhibitor against trypsin, thrombin, plasmin, factor Xa, tPA, and activated protein C. As preciously suggested that proteases may also be involved in coronavirus cellular entry, and due to the increased risk of thrombosis in COVID19, we elected to study if dabigatran can inhibit coronavirus cellular entry and replication. If yes, dabigatran can be used to combat COVID with both anti-viral and anti-thrombosis effects. Unlike camostat, dabigatran dose-dependently inhibited OC-43 virus replication as shown by RT-qPCR from the supernatant of infected cells (Fig. 3A). Compare to control, the difference of Ct (cycle threshold) is 14 cycles suggesting a 16448 fold difference in viral DNA burden between control and dabigatran treated cells. This is further confirmed by Western blot showing a significant reduction of viral protein when OC43 infected MRC-5 cells were treated with dabigatran (Fig. 3B). Even a low dose of dabigatran was able to inhibit OC43 replication in MRC-5 cells after 3 days (Fig. 3C).

Dabigatran dose-dependently improved CPE of OC43

One hour after adding the OC43 virus into the MRC-5 cells, dabigatran was added at varying doses (2.5, 50, 100 μM). Compare to control, dabigatran treated cells showed a dose-dependent decrease of CPE on day 3 and day 6 respectively (Fig. 3D and data not shown).

Dabigatran prevents OC43 entry

We next compared the OC43 replication in MRC-5 cells treated with dabigatran. For the pre-treatment, MRC-5 cells were pre-treated with dabigatran (1mM) for 1 hour before being inoculated with OC43. For the post-treatment, MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without dabigatran (1mM). The cells were collected 48h later and subjected to western blot for viral protein OC43 quantification. Western blot of the viral protein showed dabigatran treated cells (both pre-treatment and post-treatment) had much lower viral protein compare to control. More interestingly, pre-treatment of dabigatran before viral inoculation had an even lower viral load compared to cells with dabigatran after viral inoculation. This is suggestive that dabigatran may also prevent viral entry (Fig. 4A).

Dabigatran and Remdesivir synergistically inhibited coronavirus protein expression

Remdesivir is an adenosine analog that inhibits viral replication through binding to the viral RNA-dependent RNA polymerase and prematurely terminates RNA transcription. It has been approved by FDA for severe COVID-19 treatment. However, the drug supply and cost are prohibitive. It has been reported that Remdesivir demonstrated the most potent activity with EC50 and EC90 values of 0.77 and 1.76μM, respectively [15]. We decide to evaluate if dabigatran can synergistically improve the Remdesivir inhibitory effect on viral protein expression. As shown in Fig. 4B, both dabigatran and Remdesivir decreased the expression of viral protein. However, the combination of dabigatran and Remdesivir, even with 0.1μM Remdesivir, showed a significant reduction of OC43 viral replication when compared to dabigatran alone or Remdesivir alone (Fig. 4B).

Dabigatran but not camostat decreased SARS-CoV-2 in Vero E6 cells

Finally, we tested the effect of dabigatran and camostat on SARS-CoV-2 replication. As shown in Fig. 5A, Camostat (100μM) added 1 hour after SARS-CoV-2 inoculation had minimal effect on viral particle concentrations after 48 hours. However, dabigatran, on the other side (Fig. 5B), significantly decreased the viral particle concentration in the cells suggesting its inhibitory effect on SARS-CoV-2 replication.

Discussion

COVID-19 continues to be a devastating pandemic resulting in millions of deaths worldwide. Currently, multiple classes of drugs have been used to treat COVID-19, including antiviral agents, inflammation inhibitors, and convalescent plasma. However, no treatment is exceptionally effective and the availability and the cost of the agents are prohibitive.

In our study, we found Dabigatran, a protease inhibitor, dose-dependently inhibited OC43 cell entry and replication, and reduced CPE. In addition, dabigatran and Remdesivir synergistically inhibited coronavirus protein expression. These findings are important during the pandemic when effective treatment is urgently needed. Since dabigatran has been approved by FDA and its safety profile is known, it can be quickly engaged in clinical trials to save significant time avoiding going through lengthy preclinical and clinical safety testings.

Since Hoffmann et al. determined that camostat mesylate, a serine protease inhibitor, can partially block SARS-CoV cell entry mechanism [12] and significantly reduced mortality following SARS-CoV infection in animal studies [13], other serum protease inhibitors have been tried in battling this disease. Gabexate mesylate, another serine protease inhibitor, only had minimal inhibition of viral entry [16] while nafamostat mesylate, approved in Japan for acute pancreatitis and disseminated intravascular coagulation, effectively inhibits protein medication fusion in SARS-CoV-2 by targeting TMPRSS2 even at low doses (0.01 μM). [11,16] Nafamostat mesylate showed better efficacy in Calu- 3 cells with half-maximal inhibitory concentration (IC50) of 0.0022 μM, which is approximately 600-fold more potent than Remdesivir with an IC50 of 1.3 μM [17]. In addition, Nafamostat has also a role in DIC treatment which is beneficial in SARS-CoV 2 patients, however, its short half-life and intravenous administration limits its use [16]. In our study, we found dabigatran, another protease inhibitor, not only acts at the level of virus entry but also reduces the virus replication in the cell. It has been shown that initial lower viral inoculation resulting in fewer and shorter symptoms as well as a lower likelihood of viral shedding [18,19] while a greater viral load is related to more severe illness [20–22]. Therefore, since dabigatran works at the very early stage of viral infection, it may reduce the intracellular viral load and prevent the disease and potentially decrease the severity of the disease. However, the exact mechanism of how dabigatran works on coronavirus cellular entry and replication needs further investigation. Trypsin-induced cleavage of the S protein has been reported in HCoV-229E for cell entry. [23] Additionally, studies have shown that trypsin is effective in inducing fusion of

SARS-COV- infected VeroE6 cells and facilitating vial entry from the cell surface, a more efficient infection than entry through endosome[24,25]. In addition to trypsin and TMPRSS-2, furin, cathepsins, TMPRSS-4, or human airway trypsin-like protease have all been shown to leave coronavirus S proteins. Whether dabigatran has any effect on those proteases remains elusive and whether dabigatran affects viral protease also remains unknown.

In our study, we also showed the synergistic antiviral effect between the dabigatran and Remdesivir even at a very low drug dose. Due to the high demand for remdesivir and limited supply, medication rationalization has been utilized. In light of our findings, there is a possibility even a lower dose remdesivir can achieve effective antiviral effects when used in combination with widely available dabigatran. By doing this, even with the limited availability, remdesivir can be used in more patients to save more lives. Additionally, by using the combination of a lower dose of remdesivir and the dabigatran, the cost of the treatment will also decrease dramatically.

In addition, dabigatran, an FDA-approved anticoagulant can prevent thromboembolic events through direct anti-thrombin effects. COVID-19 is a hypercoagulable status with a significant risk of thromboembolic events. In a recent meta-analysis, the overall venous thrombotic event rate was 21%, with deep vein thrombosis rate of 20%, pulmonary embolism rate of 13%, while arterial thrombotic event rate was 2%. Thromboembolism significantly increased the odds of mortality by as high as 74% (OR, 1.74; 95%CI, 1.01–2.98; P = 0.04) [26]. In another study, 71.4% of fatal cases but only 0.6% of the surviving patients had disseminated intravascular coagulation. [27] To mitigate the prothrombotic state associated with COVID-19, the International Society of Thrombosis and Hemostasis (ISTH) recommends “prophylactic dose low molecular weight heparin should be considered in all patients (including non-critically ill) who require hospital admission for COVID-19 infection” in the absence of contraindications [28] and several other consensus statements, guidelines and reviews have also made similar recommendations of thromboprophylaxis for COVID-19 patients, especially for hospitalized patients [29–31]. Initially, at the start of the pandemic, several organizational guidelines suggested switching direct oral anticoagulants to unfractionated heparin low molecular weight heparin concerning possible drug-to-drug interactions, especially with antiviral agents remdesivir which inhibits CYP3A4. [31–34] However, unlike apixaban and rivaroxaban, which undergo CYP3A4 metabolism, dabigatran doesn't metabolize through the CYP450 system and therefore has few drug-drug and drug-food interactions.[35] In addition, dabigatran has a very broad safety concentration, even with increased drug concentration with remdesivir, it is still likely to be a safe range. Another advantage of dabigatran is it has a specific antidote that rapidly reveres its bleeding-related complications.[36] Therefore, dabigatran has been proposed as a first-line oral anticoagulation choice for COVID-19 patients with nonvalvular atrial fibrillation upon discharge.[37]

In conclusion. our study showed that protease inhibitor dabigatran can prevent coronaviral entry and replication and potentially can be used to treat COVID19. In addition, Dabigatran has shown synergistic inhibition on virus replication we used with the remdesivir together. This finding is significant since COVID-19 is a hyper-coagulable status with a significant risk of thrombosis event which can be

simultaneously treated with the function of dabigatran, an FDA-approved anticoagulant due to its anti-thrombin effects. It is reasonable to consider a clinical trial to evaluate the effectiveness of dabigatran in treating COVID-19 patients utilizing both anti-viral and anti-thrombin effects. Additionally, the synergistic inhibitor effect of dabigatran and Remdesivir may decrease the dose needed for COVID-19 treatment, therefore, minimize the shortage of medication, provide treatment for more patients, and decrease cost.

Material And Methods

Dabigatran was purchased from Toronto Research Chemicals (Toronto, ON, Canada, Cat: D100150). Camostat was purchased from Santa Cruz Biotechnology (Santa Cruz, Cal, sc-203867). Remdesivir was obtained from Cayman Chemicals (Ann Arbor, MI, Cat # 30354).

Cell culture

MRC-5 (ATCC, Manassas, VA, CCL-171, human lung cell) was cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, 30-2003). HCT-8 (ATCC, CCL-244, human colon cancer cell), and Huh-7 (JCRB cell bank, JCRB0403, human liver cancer cell) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; 10082–147, Gibco, USA) and penicillin G sodium 100 units/mL, streptomycin sulfate 100 ug/mL and amphotericin B 250 ng/mL (antibiotic-antimycotic; 15240–062, Gibco, USA) at 37°C with 5% CO2. Human coronavirus OC43 was obtained from ATCC (VR-1558). SARS-CoV-2 was isolated from a Chinese patient and cultured in Vero E6 cells.

Virus infection

To study the effect of protease inhibitors on viral replication, cells were infected with OC43 and SARS-CoV-2 virus for one hour before treated with camostat (25μM-100μM) or dabigatran (1μM-10μM) for the indicated time.

Quantitative RT-PCR of OC43

To evaluate the replication of OC43, the same aliquot of the virus was inoculated onto HCT-8 cells. After incubation for 1 h, the cells were washed twice and incubated for 48h before cell supernatants were obtained to evaluate virus replication. For the entry inhibition assay using protease inhibitors, viruses were inoculated onto cells that had been pre-treated with camostat mesylate or Dabigatran for 1 h and then incubated for 48h before cells were collected to evaluate virus replication.

RNA was extracted from supernatant from infected cells using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, Cat#52906). RNA (1 μg) was reverse transcribed to complementary DNA using the Go Script RT kit (Promega, Madison, WI, Cat# A5003). qPCR was done using PowerUp SYBR Green Master Mix (Applied Biosystems, BEDFORD, MA, Cat# A25742;) as described previously. [38] The forward primer

for OC43: 5'- GTTAGGCCGATAATTGAGGACT -3'; the reverse primer for OC43: 5'-ATGTAAAGATGGCCGCGTA-3'.

Western blot

Total cell lysates or cell supernatant were prepared using SDS-containing sample buffer and then subjected to SDS-PAGE before transferred onto a PVDF membrane. The blots were incubated with primary antibodies and processed using standard protocols. The monoclonal Coronavirus OC43 antibody (Cat#MAB-9013; 1:1000) and anti-GAPDH rabbit polyclonal antibody (Cat# G8795; 1:5000) were purchased from Millipore Sigma (Burlington, MA).

Cytopathic Effects

The cytopathic effects were recorded by the Olympus IX71 microscope at the indicated time after infection.

Immunofluorescence

An indirect immunofluorescence assay was used to detect SARS-CoV-2. Briefly, Vero E6 cells infected with SARS-CoV-2 were washed and fixed with 5% paraformaldehyde 48 h post-inoculation. Cells were permeabilized and incubated with homemade antibodies against SARS-CoV-2 for 30 min at 37°C before cells were washed and fluorescent-labelled anti-IgG was added. The cells were then observed under an Olympus fluorescence microscope. This experiment was performed by Dr. Ping Zhao from Changhai Hospital, Shanghai, China in a BL3 level lab.

Abbreviations

COVID-19: Coronovirus-19

SARS CoV: Severe Acute Respiratory Syndrome coronavirus

MERS-CoV: Middle East Respiratory Syndrome coronavirus

CPE: cytopathic effect

TMPRSS2: transmembrane protease/serine subfamily member 2

Declarations

Author contributions:

YB, BJ conceived and drafted the study. BJ collected all data. BJ and YB analyzed and interpreted the data. ADN, YB, BJ drafted the manuscript. All authors have approved the final draft of the manuscript.

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Figures

Fig 1

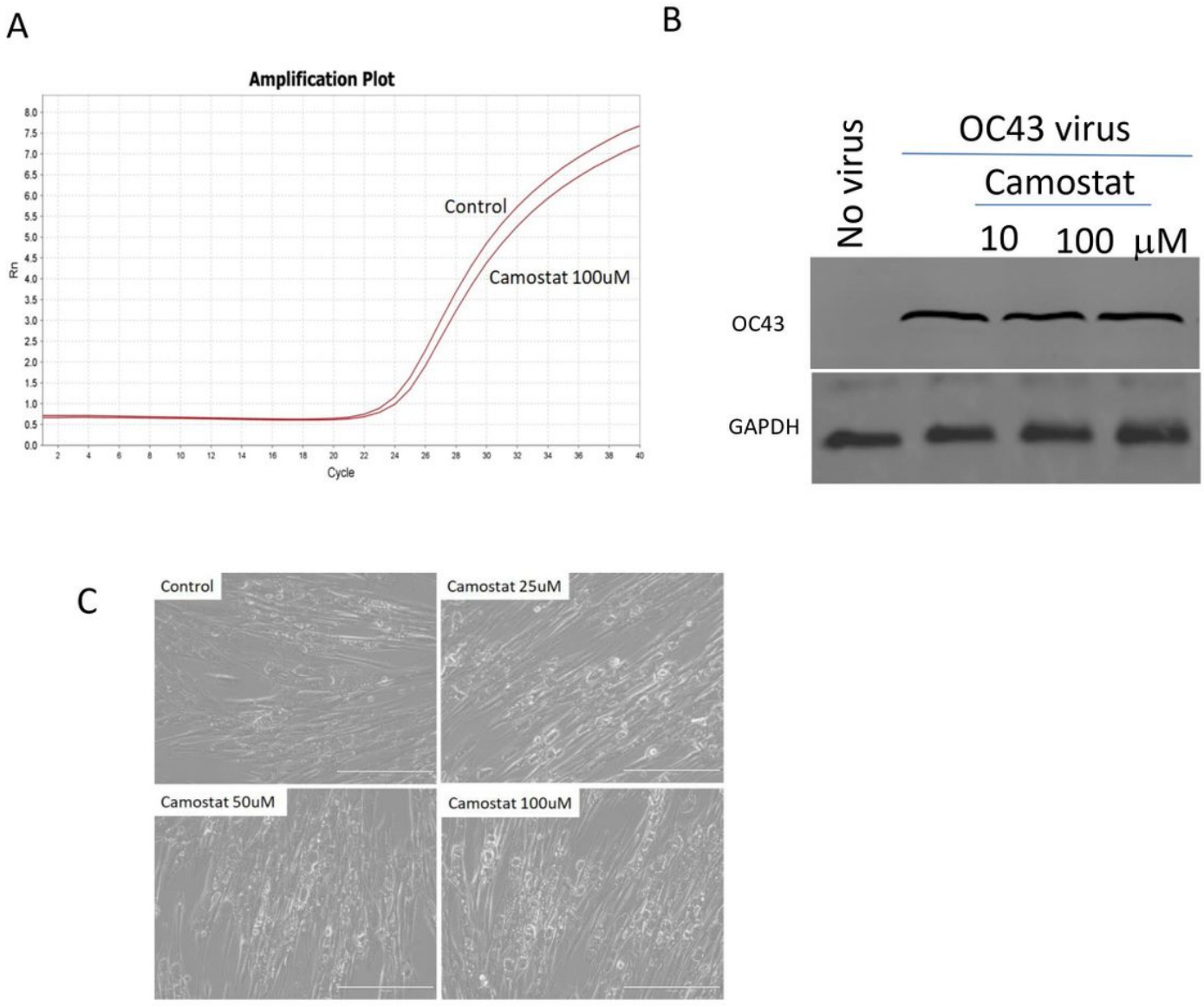

Figure 1

1A: q-RT-PCR showed Camostat had no effect on OC43 virus replication. MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without Camostat (100μM). The supernatant was collected 48 h later and subjected to q-RT-PCR for viral load quantification. 1B: Camostat had no effect on OC43 viral protein level. MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without Camostat (100μM). The cells were collected 48 h later and subjected to western blot for viral protein OC43 quantification. 1C: Camostat has no effect on CPE in MRC-5 cells on day 3. MRC5 cells were inoculated OC43 for 1 hour and then incubated with indicated concentrations of camostat for 3 days and CPE was evaluated under a light microscope. Representative micrographs were shown.

Fig 2

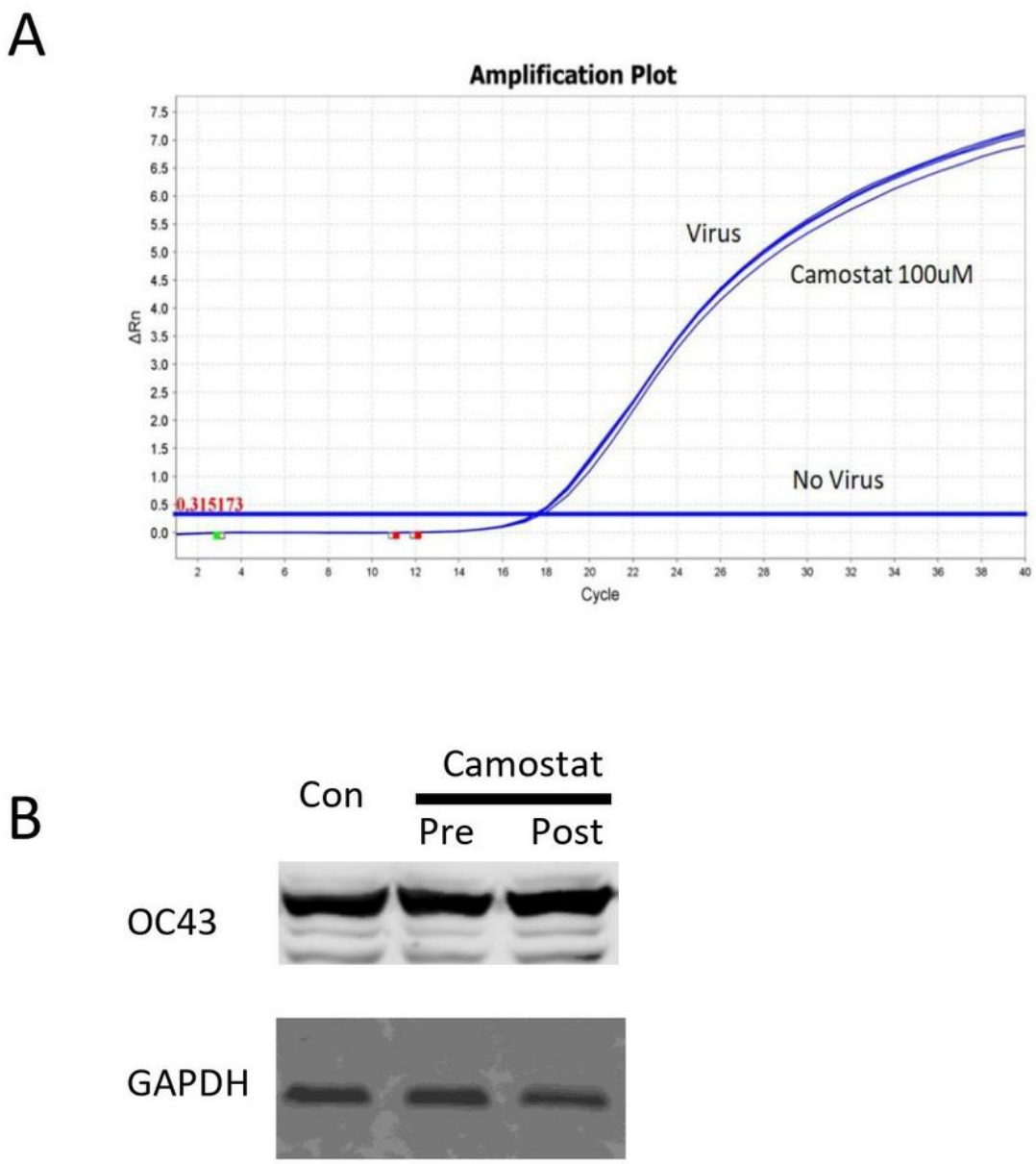

Figure 2

2A: q-RT-PCR showed Camostat had no effect on OC43 virus entry. MRC-5 cells were pre-treated with Camostat (100μM) for 1 hour before being inoculated with OC43. The supernatant was collected 48h later and subjected to q-RT-PCR for viral load quantification. 2B: Camostat had no effect on OC43of viral protein level. For the pre-treatment, MRC-5 cells were pre-treated with Camostat (100μM) for 1 hour before being inoculated with OC43. For the post-treatment, MRC-5 cells were inoculated with OC43 for 1 hour

before the medium was changed to containing with or without Camostat (100μM). The cells were collected 48h later and subjected to western blot for viral protein OC43 quantification.

Fig 3

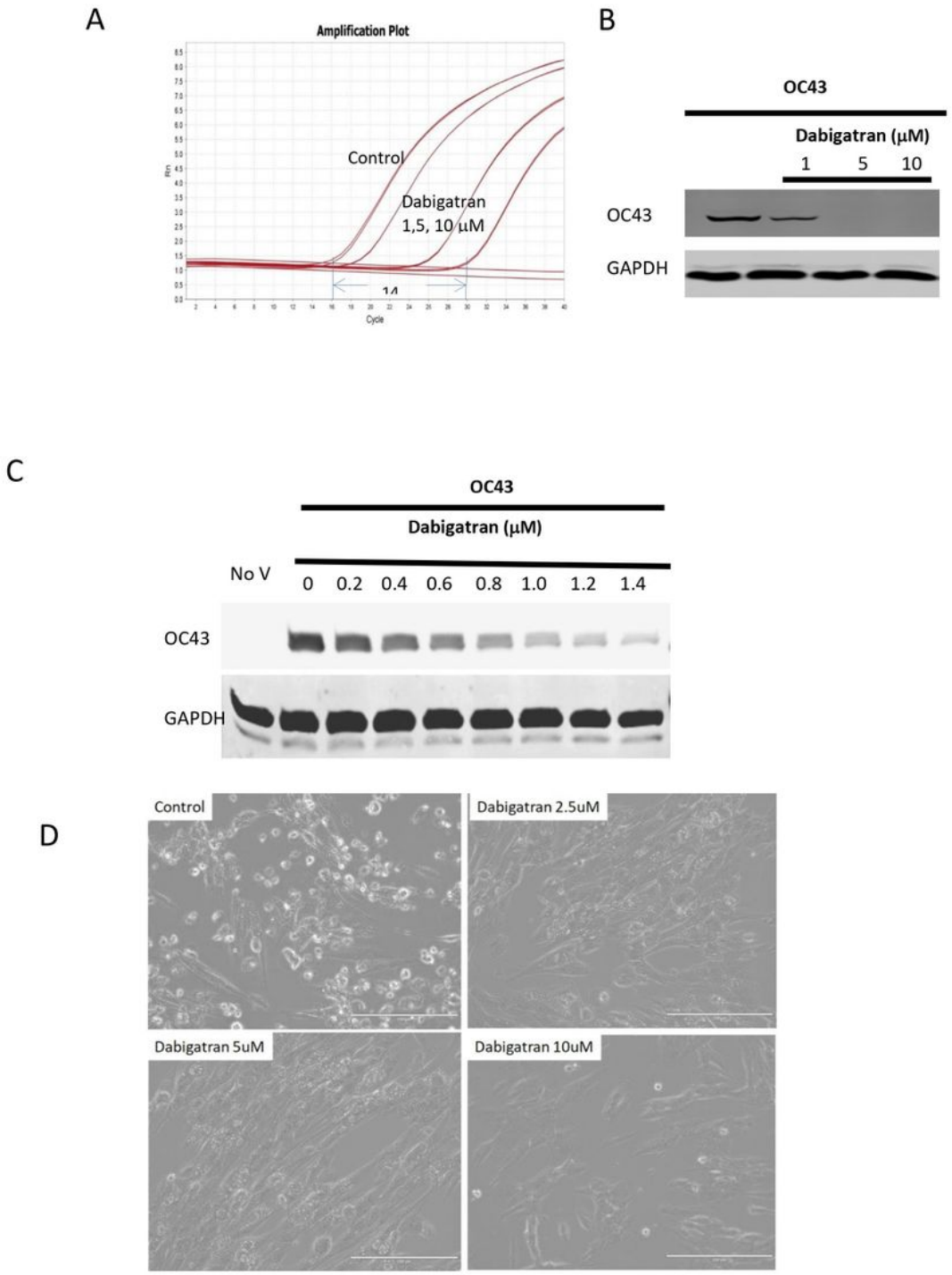

Figure 3

3A: Dabigatran dose-dependently inhibits OC43 virus production. MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without dabigatran (1, 5, or 10 μM). The supernatant was collected 48h later and subjected to q-RT-PCR for viral load quantification. Note: each

cycle difference equals 2-fold change (214=16448 fold at 10μM) 3B: Dabigatran dose-dependently abolished OC43 protein expression. MRC-5 lung cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without different concentrations of the dabigatran. The cells were collected 48 h later and subjected to western blot for viral protein OC43 quantification. GAPDH is used as a loading control. 3C: A lower dose of dabigatran was able to inhibit OC43 replication. MCR-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without different concentrations of the dabigatran (0.2-1.4mM). The cells were collected 48 h later and subjected to western blot for viral protein OC43 quantification. GAPDH is used as a loading control. 3D: Dabigatran dose-dependently (2.5, 5, 10 μM) improve CPE in MRC-5 cells. MRC5 cells were inoculated OC43 for 1 hour and then incubated with indicated concentrations of dabigatran for 3 days and CPE was evaluated under a light microscope. Representative micrographs were shown.

Fig 4

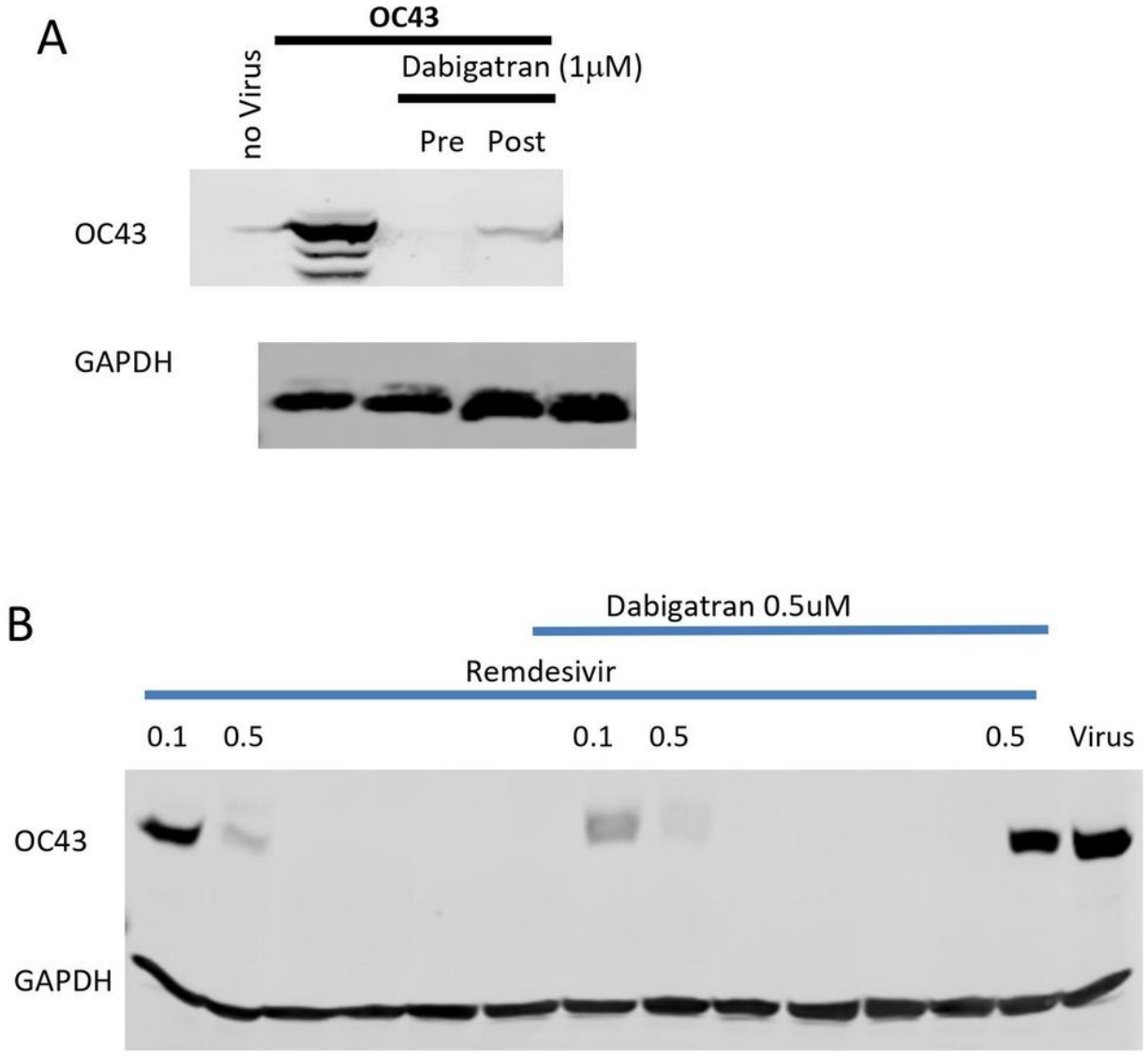

Figure 4

4A: Dabigatran pre-treatment had better viral inhibition compare to post-treatment on OC43 protein expression. For the pre-treatment, MRC-5 cells were pre-treated with dabigatran (1μM) for 1 hour before being inoculated with OC43. For the post-treatment, MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without dabigatran (1μM). The cells were collected 48h later and subjected to western blot for viral protein OC43 quantification. 4B: Dabigatran and

Remdesivir synergistically inhibited coronavirus protein expression MRC-5 cells were inoculated with OC43 for 1 hour before the medium was changed to containing with or without dabigatran (0.5μM) and Remdesivir (0.1 and 0.5μM). The cells were and subjected to western blot for viral protein OC43 quantification. Western blot of the viral protein showed both dabigatran and Remdesivir treated cells had lower viral proteins. However, the combination of dabigatran and Remdesivir showed synergistic inhibitory effects on OC43 expression than either drug alone.

Fig 5

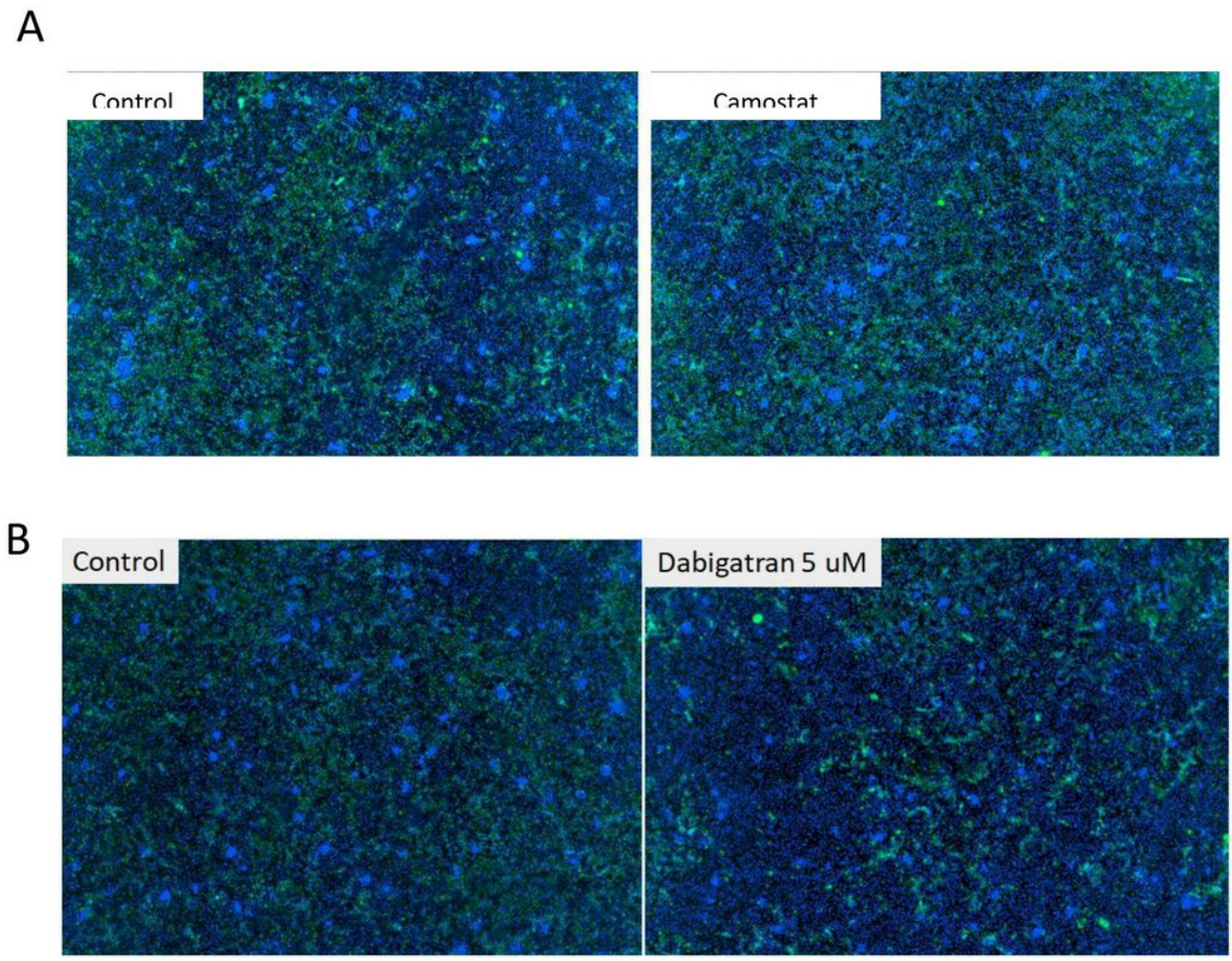

Figure 5

5A: Immunofluorescent of SARS-CoV-2 showed camostat did not have any effect on SARS-CoV-2. Camostat pre-treated Vero E6 cells were inoculated with SARS-CoV-2 for 48h before washed and fixed with 5% paraformaldehyde. Cells were permeabilized and incubated with antibodies against SARS-CoV-2 for 30 min and then fluorescent-labelled anti-IgG. Representative micrographs were shown. 5B:

Dabigatran on SARS-CoV-2 Immunofluorescence. Dabigatran pre-treated Vero E6 cells were inoculated with SARS-CoV-2 for 48h before washed and fixed with 5% paraformaldehyde. Cells were permeabilized and incubated with antibodies against SARS-CoV-2 for 30 min and then fluorescent-labelled anti-IgG. Representative micrographs were shown.