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Protease Inhibitors: Candidate Drugs to Inhibit Severe Acute Respiratory Syndrome Coronavirus 2 Replication

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The number of patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly increased, although the WHO declared a pandemic. However, drugs that function against SARS-CoV-2 have not been established. SARS-CoV-2 has been suggested to bind angiotensin-converting enzyme 2, the receptor of the SARS coronavirus. SARS coronavirus and coronavirus 229E, the cause of the common cold, replicate through cell-surface and endosomal pathways using a protease, the type II transmembrane protease. To examine the effects of protease inhibitors on the replication of coronavirus 229E, we pretreated primary cultures of human nasal epithelial (HNE) cells with camostat or nafamostat, each of which has been used for the treatment of pancreatitis and/or disseminated intravascular coagulation. HNE cells were then infected with coronavirus 229E, and viral titers in the airway surface liquid of the cells were examined. Pretreatment with camostat (0.1-10 μg/mL) or nafamostat (0.01-1 μg/mL) reduced the titers of coronavirus 229E. Furthermore, a significant amount of type II transmembrane protease protein was detected in the airway surface liquid of HNE cells. Additionally, interferons have been reported to have antiviral effects against SARS coronavirus. The additive effects of interferons on the inhibitory effects of other candidate drugs to treat SARS-CoV-2 infection, such as lopinavir, ritonavir and favipiravir, have also been studied. These findings suggest that protease inhibitors of this type may inhibit coronavirus 229E replication in human airway epithelial cells at clinical concentrations. Protease inhibitors, interferons or the combination of these drugs may become candidate drugs to inhibit the replication of SARS-CoV-2.

Keywords: camostat; coronavirus 229E; nafamostat; SARS-CoV-2; type II transmembrane protease
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Introduction

The number of patients with coronavirus disease 2019 (COVID-19) caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly increased to more than 2.5 million (as of April 23, 2020), although the WHO declared a pandemic on March 11, 2020. Drugs against SARS-CoV-2 have not been established; however, several candidate drugs have been suggested, including the protease inhibitors lopinavir and ritonavir, the nucleotide analogue prodrug remdesivir (Grein et al. 2020), the inhaled corticosteroid ciclesonide, the 4-aminoquinolines chloroquine and hydroxychloroquine (Ferner and Aronson 2020), and the guanine derivative ribavirin that has been approved to treat infection with type C hepatitis virus and respiratory syncytial virus (Li and De Clercq 2020).

Lu et al. (2020) demonstrated that the genomic charac-

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terization of SARS-CoV-2 is similar to that of severe acute respiratory syndrome (SARS) coronavirus and suggested that SARS-CoV-2 can bind angiotensin-converting enzyme 2 (ACE2), the receptor of the SARS coronavirus (Huang et al. 2006). The ACE2 is an enzyme which converts angiotensin II to angiotensin 1-7 (Burrell et al. 2004) and is abundantly expressed in alveolar type II epithelial cells (Hamming et al. 2004).

Although the replication pathways of SARS-CoV-2 are still uncertain, replication of SARS coronavirus is mediated by cell-surface and endosomal pathways using a protease, the type II transmembrane protease (Simmons et al. 2005; Bertram et al. 2012), and coronavirus 229E, the cause of the common cold, also replicates using these pathways (Kawase et al. 2009; Bertram et al. 2013). However, the development of antiviral drugs to treat SARS-CoV-2 infection is required to reduce mortality.

Kawase et al. (2012) demonstrated that the protease inhibitor camostat, which has been used for the treatment of chronic pancreatitis (Sai et al. 2010), reduced the replication of SARS coronavirus. Nafamostat has also been used for the treatment of pancreatitis and disseminated intravascular coagulation (Yamamoto et al. 2016; Minakata et al. 2019). Camostat and nafamostat are guanidinobenzoate derivatives with similar structures that inhibit protease activity (Fujii and Hitomi 1981; Ohkoshi 1981). However, the effects of camostat and nafamostat on the replication of coronavirus 229E in primary cultures of human airway epithelial cells have not been studied.

Materials and Methods

To examine the effects of protease inhibitors on the replication of coronavirus 229E, primary cultures of human nasal epithelial (HNE) cells were isolated by the treatment of nasal polyps with protease (Sigma-Aldrich, St Louis, MO, USA) and cultured on filter membranes (Transwell, Corning, Corning, NY, USA) using previously reported methods (Yamaya et al. 2020). This study was approved by

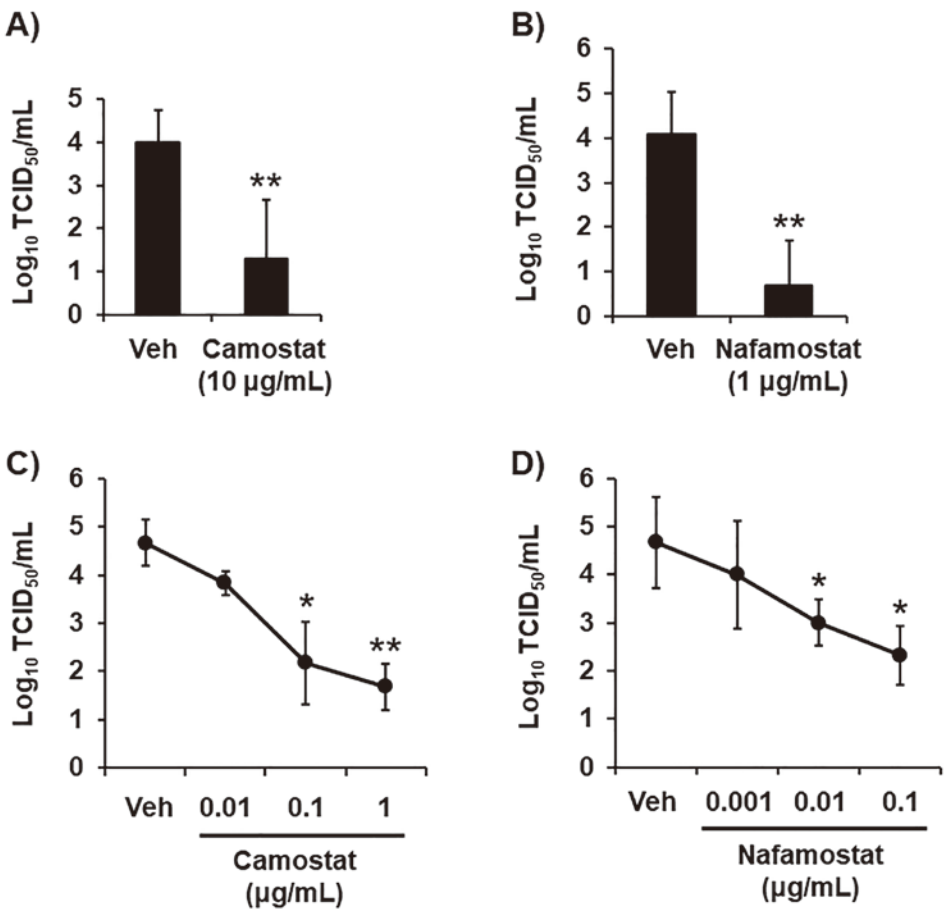

Fig. 1. Inhibitory effects of camostat and nafamostat on the release of coronavirus 229E into the airway surface liquid.
A) and B) Viral titers in the airway surface liquid of HNE cells collected between 24 h and 72 h after the infection of HNE cells pretreated with camostat (10 μg/mL) (A), nafamostat (1 μg/mL) (B) or vehicle (Veh).
C) and D) Concentration-dependent effects of pretreatment with camostat (C), nafamostat (D) or vehicle (Veh) on the release of coronavirus 229E into airway surface liquid collected between 24 h and 72 h after infection.
A-D) The results from seven (A), ten (B) or three (C and D) subjects are presented as the mean ± S.D. The viral titers are expressed as the $\log_{10}$ $TCID_{50}$/mL. Significant differences compared with cells pretreated with vehicle are indicated by *$p < 0.05$ and **$p < 0.01$.

the Tohoku University Ethics Committee (IRB number: 2018-1-15).

HNE cells were pretreated with camostat (0.01-10 μg/mL), nafamostat (0.001-1 μg/mL), or vehicle (1% double distilled water in fresh medium) for 1 h before infection until the end of the experiments. The cells were infected with coronavirus 229E and airway surface liquid was collected 24 h and 72 h after infection by rinsing the apical surfaces of HNE cells with 200 μL of fresh medium (Yamaya et al. 2020). Then, viral titers in airway surface liquid collected between 24 h and 72 h after infection was examined using endpoint methods as previously reported (Yamaya et al. 2020) and the 50% tissue culture infective dose ($TCID_{50}$) was calculated. The viral titers in airway surface liquid are expressed as $TCID_{50}$/mL.

The amount of type II transmembrane protease protein, which activates SARS coronavirus and coronavirus 229E (Bertram et al. 2012; 2013), in the airway surface liquid of uninfected HNE cells was measured using an enzyme-linked immunosorbent assay kit (TMPRSS2 ELISA kit; MyBioSourse, San Diego, CA, USA).

For comparisons of continuous variables between the two groups, Student's t-test was used. All analyses were performed using SPSS version 21 (IBM Japan, Tokyo, Japan).

Results and Discussion

Camostat and nafamostat inhibit protease activity (Fujii and Hitomi 1981; Ohkoshi 1981). Pretreatment of HNE cells with camostat (0.1-10 μg/mL) or nafamostat (0.01-1 μg/mL) reduced the titers of coronavirus 229E in the airway surface liquid of HNE cells (Fig. 1A-D). Furthermore, a significant amount of type II transmembrane protease protein was detected in the airway surface liquid of uninfected HNE cells (323 ± 110 pg/mL, n = 3, mean ± SD).

Because the maximum reported serum concentrations of camostat and nafamostat are 0.12 and 0.09 μg/mL, respectively (Abe et al. 1984; Pharmaceuticals and Medical Devices Agency 2018), these findings suggest that protease inhibitors of this type at clinical concentrations inhibit coronavirus 229E replication through inhibiting activation of the coronavirus spike protein (Kawase et al. 2012) (Fig. 2).

Second, interferons have been reported to have antiviral effects against SARS coronavirus (Spiegel et al. 2004). In addition, several previous and ongoing studies have examined the additive effects of interferons on the inhibitory effects of other candidate drugs of SARS-CoV-2 infection, such as lopinavir, ritonavir and favipiravir, on the replication of SARS-CoV and Middle East respiratory syndrome coronavirus (Li and De Clercq 2020; Martinez 2020; Sheahan et al. 2020). Furthermore, interferons have been used for the treatment of type C hepatitis in combination with nonsteroidal anti-inflammatory drugs to modulate high temperature (Muñoz et al. 2000).

Thus, protease inhibitors, interferons or the combination of these drugs may become candidate drugs to inhibit the replication of SARS-CoV-2, although we did not study the effects of these drugs on the replication of SARS-CoV-2. Animal and clinical studies are required to confirm the efficacy and adverse effects of these treatments.

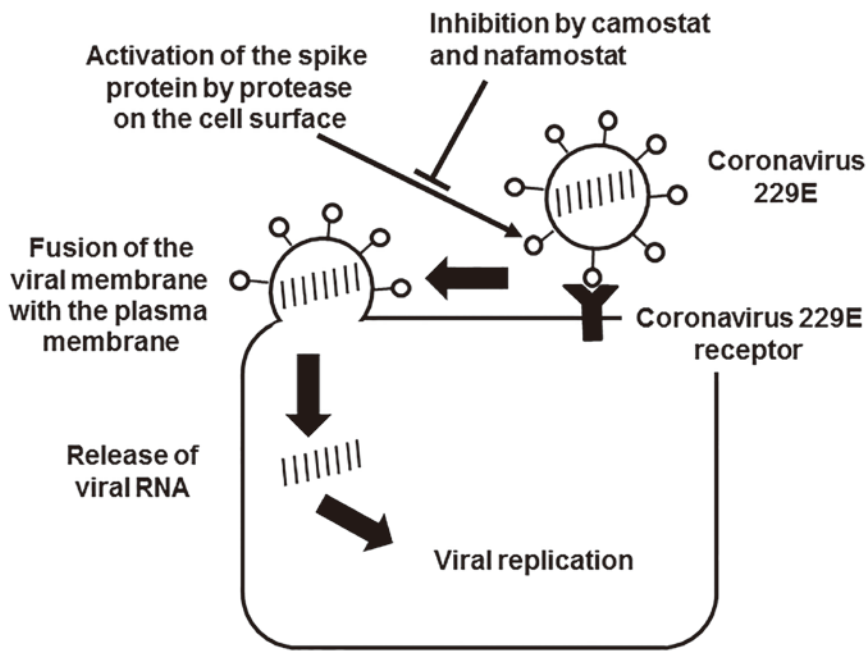

Fig. 2. The site of camostat and nafamostat action in the coronavirus 229E replication pathway.
Type II transmembrane protease, which is expressed in HNE cells, activates the spike protein of coronavirus 229E, which attaches to the receptor on the plasma membrane. Then, the viral membrane fuses with the plasma membrane, and subsequent steps, including RNA release into the cytoplasm and viral replication, occur.

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Conflict of Interest

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References

Abe, T., Kinoshita, T., Matsuda, J., Ogushi, T., Kawasugi, K., Yoshimura, Y. & Otomo, M. (1984) Phase I study of FUT-175: single and multiple dose study. *Jpn. Pharmacol. Ther.,* **12**, 4941-4964 (in Japanese).

Bertram, S., Dijkman, R., Habjan, M., Heurich, A., Gierer, S., Glowacka, I., Welsch, K., Winkler, M., Schneider, H., Hofmann-Winkler, H., Thiel, V. & Pohlmann, S. (2013) TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. *J. Virol.*, **87**, 6150-6160.

Bertram, S., Heurich, A., Lavender, H., Gierer, S., Danisch, S., Perin, P., Lucas, J.M., Nelson, P.S., Pohlmann, S. & Soilleux, E.J. (2012) Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. *PLoS One*, **7**, e35876.

Burrell, L.M., Johnston, C.I., Tikellis, C. & Cooper, M.E. (2004) ACE2, a new regulator of the renin-angiotensin system. *Trends Endocrinol. Metab.*, **15**, 166-169.

Ferner, R.E. & Aronson, J.K. (2020) Chloroquine and hydroxychloroquine in covid-19. *BMJ*, **369**, m1432.

Fujii, S. & Hitomi, Y. (1981) New synthetic inhibitors of C1r, C1 esterase, thrombin, plasmin, kallikrein and trypsin. *Biochim. Biophys. Acta*, **661**, 342-345.

Grein, J., Ohmagari, N., Shin, D., Diaz, G., Asperges, E., Castagna, A., Feldt, T., Green, G., Green, M.L., Lescure, F.X., Nicastri, E., Oda, R., Yo, K., Quiros-Roldan, E., Studemeister, A., et al. (2020) Compassionate use of remdesivir for patients with severe covid-19. *N. Engl. J. Med.*, doi: 10.1056/NEJMoa2007016 [Epub ahead of print].

Hamming, I., Timens, W., Bulthuis, M.L., Lely, A.T., Navis, G. & van Goor, H. (2004) Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J. Pathol.*, **203**, 631-637.

Huang, I.C., Bosch, B.J., Li, F., Li, W., Lee, K.H., Ghiran, S., Vasilieva, N., Dermody, T.S., Harrison, S.C., Dormitzer, P.R., Farzan, M., Rottier, P.J. & Choe, H. (2006) SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. *J. Biol. Chem.*, **281**, 3198-3203.

Kawase, M., Shirato, K., Matsuyama, S. & Taguchi, F. (2009) Protease-mediated entry via the endosome of human coronavirus 229E. *J. Virol.*, **83**, 712-721.

Kawase, M., Shirato, K., van der Hoek, L., Taguchi, F. & Matsuyama, S. (2012) Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. *J. Virol.*, **86**, 6537-6545.

Li, G. & De Clercq, E. (2020) Therapeutic options for the 2019 novel coronavirus (2019-nCoV). *Nat. Rev. Drug Discov.*, **19**, 149-150.

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., et al. (2020) Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*, **395**, 565-574.

Martinez, M.A. (2020) Compounds with therapeutic potential against novel respiratory 2019 coronavirus. *Antimicrob. Agents Chemother.*, **64**, pii: e00399-20.

Minakata, D., Fujiwara, S.I., Ikeda, T., Kawaguchi, S.I., Toda, Y., Ito, S., Ochi, S.I., Nagayama, T., Mashima, K., Umino, K., Nakano, H., Yamasaki, R., Morita, K., Kawasaki, Y., Sugimoto, M., et al. (2019) Comparison of gabexate mesilate and nafamostat mesilate for disseminated intravascular coagulation associated with hematological malignancies. *Int. J. Hematol.*, **109**, 141-146.

Muñoz, A.E., Levi, D., Podesta, A., Gorin, J.M., Gonzalez, J., Bartellini, M.A., Munne, M.S., Cabanne, A., Flichman, D. & Terg, R. (2000) Interferon-alpha 2b combined with daily ketoprofen administration improves virological response in chronic hepatitis C: a prospective and randomised trial. *Gut*, **46**, 427-431.

Ohkoshi, M. (1981) Inhibition of growth of 3-methylcholanthrene-induced mouse skin tumor by protease inhibitor [N,N-dimethylcarbamoylmethyl 4-(4-guanidinobenzoyloxy)-phenylacetate] methanesulfate. *Gan*, **72**, 959-964.

Pharmaceuticals and Medical Devices Agency (2018) Approved-information of camostat mesilate. http://www.pmda.go.jp/PmdaSearch/iyakuSearch/ [*Accessed*: April 23, 2020]) (in Japanese).

Sai, J.K., Suyama, M., Kubokawa, Y., Matsumura, Y., Inami, K. & Watanabe, S. (2010) Efficacy of camostat mesilate against dyspepsia associated with non-alcoholic mild pancreatic disease. *J. Gastroenterol.*, **45**, 335-341.

Sheahan, T.P., Sims, A.C., Leist, S.R., Schafer, A., Won, J., Brown, A.J., Montgomery, S.A., Hogg, A., Babusis, D., Clarke, M.O., Spahn, J.E., Bauer, L., Sellers, S., Porter, D., Feng, J.Y., et al. (2020) Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat. Commun.*, **11**, 222.

Simmons, G., Gosalia, D.N., Rennekamp, A.J., Reeves, J.D., Diamond, S.L. & Bates, P. (2005) Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proc. Natl. Acad. Sci. USA*, **102**, 11876-11881.

Spiegel, M., Pichlmair, A., Muhlberger, E., Haller, O. & Weber, F. (2004) The antiviral effect of interferon-beta against SARS-coronavirus is not mediated by MxA protein. *J. Clin. Virol.*, **30**, 211-213.

Yamamoto, T., Yamamura, H., Yamamoto, H. & Mizobata, Y. (2016) Comparison of the efficacy of continuous i.v. infusion versus continuous regional arterial infusion of nafamostat mesylate for severe acute pancreatitis. *Acute Med. Surg.*, **3**, 237-243.

Yamaya, M., Nishimura, H., Deng, X., Sugawara, M., Watanabe, O., Nomura, K., Shimotai, Y., Momma, H., Ichinose, M. & Kawase, T. (2020) Inhibitory effects of glycopyrronium, formoterol, and budesonide on coronavirus HCoV-229E replication and cytokine production by primary cultures of human nasal and tracheal epithelial cells. *Respir. Investig.*, **58**, 155-168.