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LETTER TO THE EDITOR

The Physiological TMPRSS2 Inhibitor HAI-2 Alleviates SARS-CoV-2 Infection

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The largest disease pandemic in modern human history, caused by severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2), is still ongoing. The disease severity varies greatly (from asymptomatic to death) among individuals and by age (1, 2), but the mechanisms for the variation remain unclear. SARS-CoV-2 uses host proteases to activate its spike protein (3). The type II transmembrane serine protease TMPRSS2 plays an important role for spike protein activation (3–5). A recent study demonstrated that hepatocyte growth factor activator inhibitor 2 (HAI-2) is a cognate inhibitor of TMPRSS2 (6). Albeit less efficiently, HAI-1 also inhibits TMPRSS2 (6). In this study, the potential role of HAI-2 in inhibiting SARS-CoV-2 infection was analyzed. The His-tagged full-length ectodomain of HAI-2 was expressed in HEK293S cells lacking *N*-acetylglucosaminyltransferase I (293S GnTI$^-$ cells) and purified by Ni^{2+}-NTA affinity column and Superdex 200 GL 10/300 gel filtration chromatography, as reported previously (7). Individual fractions were then tested for trypsin inhibition activity using a Pierce Fluorescent protease assay kit, and the fraction (fraction 18–20) that contained HAI-2 proteins with an expected size (~27 kDa) and showing the highest activity was used for subsequent assays. SARS-CoV-2 may use TMPRSS2 at the plasma membrane (3–5) or lysosomal cathepsin L (8) for spike protein activation. Nafamostat and E-64d inhibit TMPRSS2- and cathepsin L-mediated coronavirus entry, respectively (9, 10). VeroE6 and VeroE6/TMPRSS2 cells (4) were infected with the SARS-CoV-2 Wk521 strain (4) in the presence or absence of inhibitors (10 μM nafamostat, 10 μM E-64d, or 20 μg/ml HAI-2), and the viral RNA levels at 6 h postinfection were quantified by real-time RT-qPCR as reported previously (4). In VeroE6/TMPRSS2 cells both TMPRSS2- and cathepsin L-mediated entry pathways are used, while in VeroE6 cells only the cathepsin L-mediated pathway is available. As expected, E-64d, but not nafamostat, blocked SARS-CoV-2 infection of VeroE6 cells, while neither nafamostat nor E-64d alone blocked SARS-CoV-2 infection of VeroE6/TMPRSS2 cells (Fig. 1A). The combined use of nafamostat and E-64d efficiently blocked SARS-CoV-2 infection of VeroE6/TMPRSS2 cells (viral RNA level reduced by ~100-fold). In this experimental condition, HAI-2 showed comparable inhibitory ability to nafamostat (Fig. 1A). Dose-dependent inhibition of SARS-CoV-2 infection in VeroE6/TMPRSS2 cells by HAI-2 in the presence of 10 μM E-64d was also observed (Fig. 1B). HAI-2 is endogenously expressed in many cell types (11) and may thus inherently inhibit or alleviate SARS-CoV-2 infection. Expression of HAI-2 in human lung adenocarcinoma Calu-3 cells was knocked down (KD) by small interfering RNA (siRNA) transfection (Fig. 1C), and virus infection assays were performed. In a previous study using Middle East respiratory syndrome coronavirus, it was

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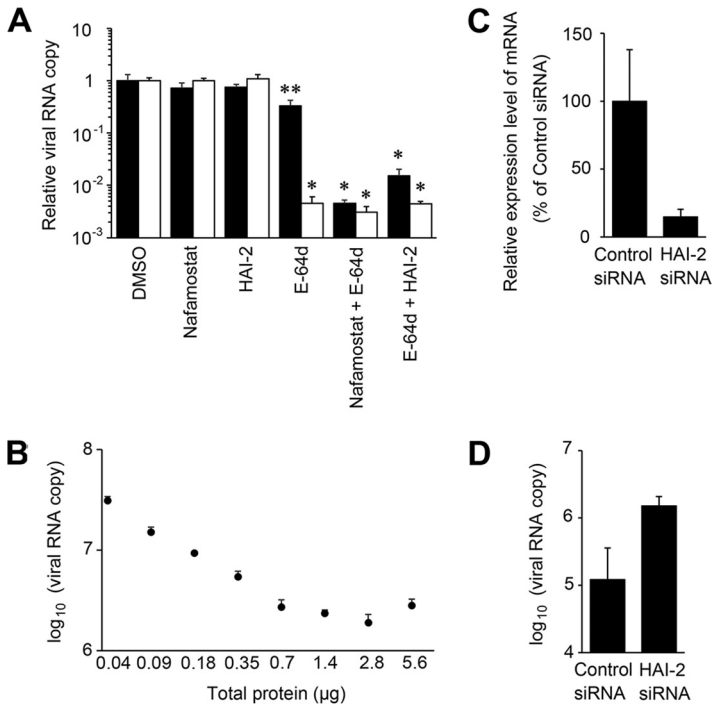

FIG 1 SARS-CoV-2 infection inhibition assay results. (A and B) VeroE6/TMPRSS2 or VeroE6 cells treated with protease inhibitors were infected with SARS-CoV-2 at an MOI of 0.1 and the viral RNA levels at 6 h postinfection were quantified by real-time RT-PCR. (A) Black and white bars indicate data with VeroE6/TMPRSS2 and VeroE6, respectively. The asterisk and double asterisk indicate $P < 0.001$ and $P < 0.01$, respectively. (B) Dose-dependent inhibition of SARS-CoV-2 infection in VeroE6TMPRSS2 cells by HAI-2 in the presence of 10 μM E-64d. (C) Effect of HAI-2 KD by siRNA transfection in Calu-3 cells. (D) Virus infection assay results in HAI-2 KD and control cells. Calu-3 cells transfected with HAI-2-specific siRNA or control siRNA were infected with SARS-CoV-2 at an MOI of 10 and the viral RNA levels at 3 days postinfection were quantified by real-time RT-PCR.

demonstrated that TMPRSS2 is mainly utilized during virus entry into Calu-3 cells and perhaps into the lung as well (12). The level of viral RNA in HAI-2 KD cells was ~40 times greater than that in control siRNA-transfected cells (Fig. 1D). These data indicated that the endogenous level of HAI-2 in Calu-3 cells alleviated SARS-CoV-2 infection. The present study provides two key messages. First, the expression level of HAI-2 modulates the infection level of SARS-CoV-2. Because SARS-CoV-2 spreads systemically *in vivo* (3), even a small imbalance or change in TMPRSS2 and HAI-2 expression may modulate tissue tropism or disease severity caused by SARS-CoV-2. Second, studies on HAI-2 may open a way to develop protein-based therapeutics against SARS-CoV-2, as already suggested for the treatment of TMPRSS2-mediated malignant tumor invasions (6, 11).

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