Alpha 1 Antitrypsin is an Inhibitor of the SARS-CoV2–Priming Protease TMPRSS2

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Abstract

The transmembrane serine protease TMPRSS2 is indispensable for S protein priming of the MERS, SARS-CoV, and SARS-CoV2 coronaviruses, a process that is necessary for entry of the virus into host cells. Therefore, inhibiting TMPRSS2 holds promise as an approach toward preventing transmission of coronaviruses. Herein, we developed an in vitro system to measure TMPRSS2 activity and tested the inhibition of TMPRSS2 by several synthetic and natural protease inhibitors. Camostat mesylate and bromhexine hydrochloride (BHH) inhibited TMPRSS2 proteolytic function. In addition, we identified the small molecule 4-(2-aminomethyl)benzenesulfonyl fluoride (AEBSF) and the human, anti-inflammatory protein alpha 1 antitrypsin (A1AT) as inhibitors of TMPRSS2. AEBSF and A1AT inhibited TMPRSS2 activity in a dose-dependent manner. AEBSF and A1AT inhibited TMPRSS2 in the same range of concentrations (100-0.1 μM). We suggest that treatment with these inhibitors, particularly A1AT, which is an FDA-approved drug, might be effective in limiting SARS-CoV and SARS-CoV2 transmissibility and as a COVID-19 treatment.

Introduction

The COVID-19 pandemic is caused by the severe acute respiratory syndrome (SARS) - coronavirus (CoV) 2. The efficient transmission of this virus has led to exponential growth in the number of worldwide cases. The RNA sequence of SARS-CoV2 (*1*) provides the basis for development of diagnostic tools and intensive research on the mechanism of the viral infection and replication. Hoffmann *et al.* provided insight on the mechanism of SARS-CoV2 entry to the host cells, which is the first step in the viral replication cycle (*2*). In this study, the researchers found that similar to other coronaviruses, SARS-CoV2 uses the angiotensin-converting enzyme 2 (ACE2) receptor for entering cells via the spike (S) protein on the virus envelope. In addition, the host serine protease TMPRSS2 is essential for processing of the viral S protein, which is prerequisite for S protein–ACE2 interaction and infection of the host. Inhibition of TMPRSS2 by camostat mesylate prevents SARS-CoV2 entry into cells in vitro. (*2, 3*). Another study demonstrated that TMPRSS2 increases the entry of SARS-CoV not only by processing of the S protein, but also by processing of the host receptor ACE2 (*4*). TMPRSS2-deficient mice had decreased viral spread in the airways compared to control mice after infection with SARS-CoV (*5*).

Herein, we performed a functional screen to identify TMPRSS2 inhibitors. We compared the efficiency of TMPRSS2 inhibition by several synthetic and natural serine protease inhibitors, including drugs with known function as protease inhibitors. We identify 4-(2-aminomethyl)benzenesulfonyl fluoride (AEBSF) and alpha 1 antitrypsin (A1AT) as novel inhibitors of TMPRSS2. We suggest that by inhibiting TMPRSS2 proteolytic activity, AEBSF and A1AT can potentially inhibit S protein processing and limit SARS-CoV2.

Materials and Methods

Materials

Secretory leukocyte peptidase inhibitor (SLPI), AEBSF, and Boc-Gln-Ala-Arg-7-Amino-4-methylcoumarin (BOC-QAR-AMC) were obtained from R&D systems. Camostat mesylate was obtained from Sigma Aldrich, and bromhexine hydrochloride (BHH) was obtained from ThermoFisher. A1AT (CSL Behring, Zemaira) was a kind gift of Mark Brantly (University of Florida, Gainesville, FL).

TMPRSS2 overexpression

A PLX304 plasmid–containing human *TMPRSS2* open reading frame from the ORFeome Collaboration (Dana-Farber Cancer Institute, Broad Institute of Harvard and Massachusetts Institute of Technology [HsCD00435929]) was obtained from DNASU Plasmid Repository, and a control PLX304 vector was obtained from Addgene (Watertown, MA, USA).

HEK-293T cell culture and transfection

HEK-293T cells were grown in DMEM supplemented with 10% FBS and seeded in a black, 96-well plate (75,000 cells/well). The following day, cells were transfected overnight with either a control plasmid (PLX) or TMPRSS2 (PLX-TMPRSS2) via TransIT LT-1 transfection reagent (Mirus Bio) in 100 µL of OptiMEM per well. The media was replaced the next day.

TMPRSS2 activity assay

Twenty-four hours after transfection, the media was replaced with 80 µL of phosphate-buffered saline (PBS). Inhibitors or PBS alone were added to the wells in the indicated

concentrations and incubated at 25ºC for 15 minutes. The fluorogenic substrate BOC-QAR-AMC (R&D Biosystems) was then added to each well to a final concentration of 100 µM. Fluorescence (excitation 365 nm, emission 410 nm) was immediately measured every 15 minutes at 37ºC using a GloMax plate reader (Promega).

Gel electrophoresis western blotting

Protein lysates of HEK-293T cells were extracted with RIPA buffer (PIERCE) and protease inhibitor cocktail (Roche). Loading buffer (Life Technologies) was added, and samples were heated to 95°C for 5 minutes and subjected to electrophoresis in 12% NuPAGE Bis-Tris gels (Life Technologies). Gels were transferred to nitrocellulose membranes (Life Technologies) and probed with the primary antibodies rabbit anti-V5 (Bethyl Laboratories) and rabbit anti–human GAPDH (ABCAM) and subsequently with the secondary antibody IRDye 800RD goat anti-rabbit (LI-COR Biosciences). Membranes were visualized and analyzed using the Odyssey CLx system (LI-COR Biosciences).

Results

Overexpression of TMPRSS2 and measurements of proteolytic activity

We aimed to establish an experimental framework for quantifying TMPRSS2 proteolytic activity. We chose to overexpress TMPRSS2 in a human cell line, HEK-293T, commonly used for experimentation, because of its high transfectability. Western blot analysis of the cell lysates revealed a band at ~60 kD in TMPRSS2-transfected cells but not in control cells (Figure 1A). GAPDH was used as a loading control. The trypsin-like activity of the transfected cells using the fluorogenic peptide substrate BOC-QAR-AMC revealed a >2.5-fold increase in the proteolytic activity of the TMPRSS2-transfected cells compared with that of control cells ($p = 0.0002$; Figure 1B). The proteolytic activity of the TMPRSS2-transfected cells increased over time compared with that of control cells (Figure 1C). The mean proteolytic rate per minute of the TMPRSS2-transfected cells was increased by >3.5 fold compared to the proteolytic rate of control cells ($p < 0.0001$, Figure 1D). The proteolytic activity could be measured hours after addition of the substrate, and the average proteolytic activity per hour was increased by >2 fold in TMPRSS2-overexpressing cells compared with control cells ($p = 0.0008$, Figure 1E). These collective data demonstrate that overexpression of TMPRSS2 results in overproduction of functional TMPRSS2 and that the proteolytic activity of TMPRSS2 can be accurately measured.

Functional screen of TMPRSS2 inhibitors

We tested the effect of protease inhibitors on TMPRSS2 activity. As a positive control, cells were treated with camostat mesylate, a drug that has been shown to inhibit TMPRSS2 and coronavirus entry into the host cells (*2*). As expected, camostat mesylate inhibited the proteolytic activity of TMPRSS2 even at the lowest concentration of 100 nM (Figure 2A). We then tested whether SLPI would inhibit TMPRSS2. However, none of the concentrations tested inhibited TMPRSS2 proteolytic activity (Figure 2B). Next, we tested, human A1AT. A1AT inhibited TMPRSS2 proteolytic activity in a dose-dependent manner with peak effects seen at 1 µM (Figure 2C). In addition, we tested AEBSF, a drug with proteolytic inhibition activity that was shown to inhibit influenza infection in a murine model of pre-infection treatment (*6*). AEBSF inhibited TMPRSS2 proteolytic activity in a dose-dependent manner, with peak inhibition seen at concentrations of 1 µM (Figure 2D). We tested BHH, an FDA-approved drug used in a mucolytic cough suppressant that was reported to have inhibitory capacity toward TMPRSS2 (*7*). BHH inhibited TMPRSS2 proteolytic activity in a dose-dependent manner, although this drug was less potent than A1AT or AEBSF (Figure 2E). Figure 2F demonstrates the proteolytic activity rate of the tested inhibitors.

Discussion

TMPRSS2 has a role in the entry of several coronaviruses, including SARS-CoV2, SARS-CoV, and MERS-CoV, as well as several types of influenza viruses (*2, 8-12*). TMPRSS2 also functions as an oncogene in prostate cancer, in which fusion events of *TMPRSS2* and *ERG2* occur (*13*). Therefore, TMPRSS2 is a promising target for drug development. Targeting host TMPRSS2 has an advantage over targeting viral proteins because anti-virals can rapidly lose their effectiveness due to the high rate of mutations that occur in the viral genome. Targeting host proteins limits the rise of drug-resistant viruses. The obstacle in targeting human proteins is the potential risk of altering physiologic pathways. It was suggested that TMPRSS2 initiates a cascade of proteolytic activation events that regulate processing of proteins in the seminal fluid and in the lung because TMPRSS2 regulates sodium channels (*14*). Nevertheless, mice deficient in TMPRSS2 lack any obvious phenotypes, suggesting that other proteases may have redundant roles and may compensate for the loss of TMPRSS2 (*15*). Therefore, delivery of TMPRSS2 inhibitors during viral infections might be a relatively safe strategy. Although the safety of TMPRSS2 inhibition was not clinically proven yet, drugs with proteolytic inhibition activity towards TMPRSS2 are clinically available, such as camostat mesylate, nafamostat, and BHH. Herein, we reveal that A1AT, which is approved by the FDA for the treatment of A1AT deficiency, can efficiently inhibit TMPRSS2. The small molecule AEBSF inhibited TMPRSS2 as well. Therefore, our collective data suggest that delivery of TMPRSS2 inhibitors, and particularly A1AT, to patients with COVID-19 can potentially inhibit TMPRSS2-mediated S protein processing and virus entry.

Previously, A1AT was shown to inhibit the infection of H3N2 influenza A and influenza B viruses in a murine model (*8*). Notably, these viruses do not require TMPRSS2 priming, and the mechanism of A1AT function was suggested to be mediated by hepsin inhibition. To our knowledge, we are the first to demonstrate inhibition of TMPRSS2 by A1AT. A1AT is mainly synthesized in the liver and found in high concentrations in the blood under normal conditions. A1AT concentration in the blood can be increased by 6-fold as part of the acute phase of inflammation or tissue injury (*16*). As a drug, A1AT is delivered to several organs, including the lungs, where it inhibits primarily neutrophil elastase, proteinase 3, and cathepsin G. Neutrophil elastase cleaves several structural proteins in the lungs and processes several innate immune mediators. In addition, A1AT promotes clearance of apoptotic cells (*17*). Therefore, A1AT is considered to be a protective protein that inhibits tissue damage and inflammatory response. Variable mutations in the A1AT gene cause deficiency of functional protein, resulting in proteolytic lung damage and panlobular emphysema (*18*). Augmentation treatment of A1AT has been approved for the treatment of A1AT deficiency (*19*).

Increased neutrophil and decreased lymphocyte counts have been found in patients with COVID-19 with severe disease compared to those with mild disease and healthy controls (*20, 21*). Therefore, we suggest that A1AT delivery can potentially decrease the pathogenicity of COVID-19 by inhibiting the alveolar inflammatory response in addition to its role as a TMPRSS2 inhibitor. Notably, truncated forms of A1AT are significantly increased in the serum of patients with SARS compared to control patients, and the truncated A1AT levels correlate with disease severity (*22*). These findings suggest that

A1AT may be a part of a natural protective mechanism to fight coronavirus infection and acute lung disease. A1AT belongs to the super family of serine protease inhibitors (SERPIN) that inhibits serine and cysteine proteases. Proteases interact with SERPINs and, in turn, cleave the serpins' reactive center loop. This cleavage results in a conformational change of the serpins such that they form covalent bonds with target proteases and irreversibly block their activity (*23*). Therefore, the truncated A1AT observed in patients with SARS may be a result of cleavage of functional A1AT by proteases, such TMPRSS2, and other enzymes, such as neutrophil elastase.

We also found that AEBSF (also called Pefabloc® SC) inhibits TMPRSS2 activity. AEBSF is a small molecule with relatively non-specific protease inhibition properties that has been shown to decrease the levels of both the H1N1 and H7N7 nucleoproteins in the lung of influenza-infected mice (*6*). AEBSF also partially inhibits fusion of the mouse hepatitis coronavirus (*24*). AEBSF occupies the S1 pocket of trypsin-like serine proteases and leads to a covalent sulfonylation of the active site (*25*).

We suggest that treatment with the protease inhibitors camostat mesylate, A1AT, BHH, and AEBSF may be a useful antiviral strategy to fight COVID-19. These protease inhibitors have the potential to prevent SARS-CoV2 entry to host cells by inhibiting S protein priming by TMPRSS2 and binding of the virus to ACE2 (Figure 3). A1AT may be particularly effective as it has dual capacity, inhibiting TMPRSS2 (and hence viral uptake and subsequent replication) and possessing anti-inflammatory activity. We suggest that using these inhibitors may be therapeutic in conditions in which TMPRSS2 function is

pathogenic, such as in prostate cancer and several types of coronavirus and influenza infections. The ready availability of A1AT calls attention to its potential clinical use for the COVID-19 pandemic.

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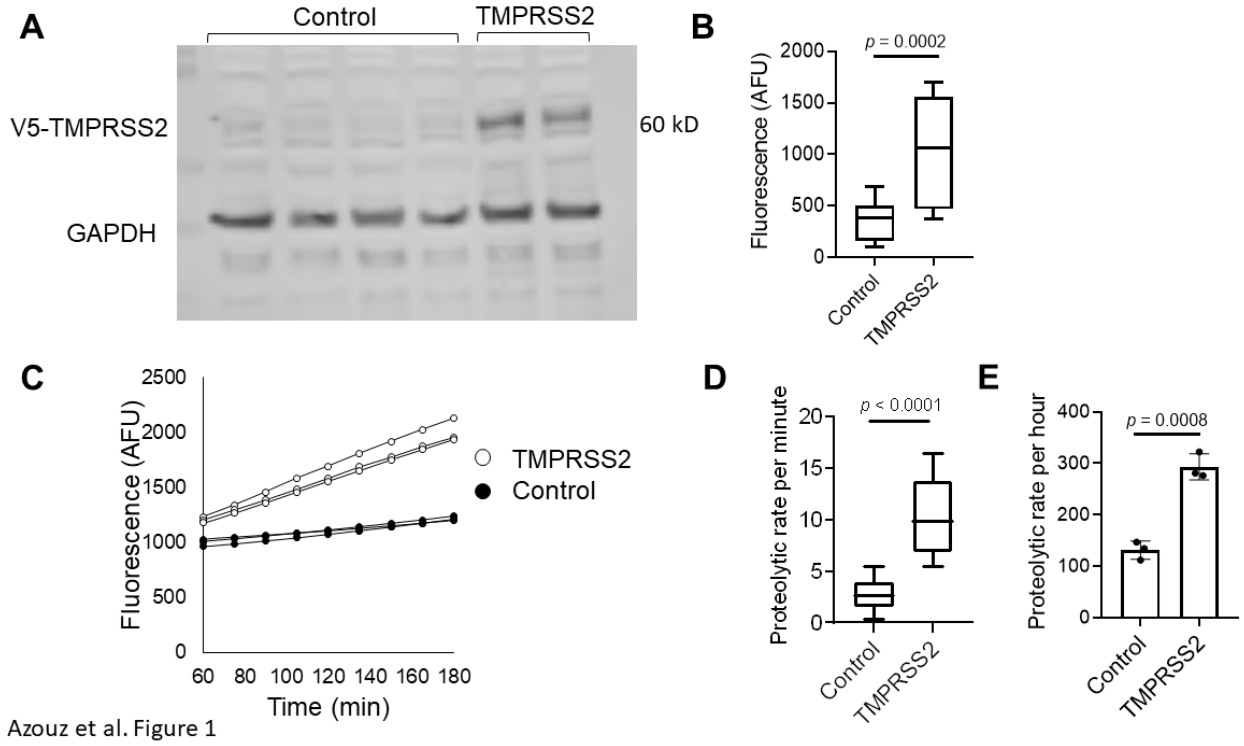

Figure 1. Measurements of TMPRSS2 activity in transfected cells. A. TMPRSS2 protein expression cells transfected with PLX304 vector or PLX304-TMPRSS2 vector assessed by Western blot. TMPRSS2-containing V5 tag was assessed by anti-V5 antibody, and anti-GAPDH antibody was used as a loading control. **B.** Arbitrary fluorescence unit (AFU) measurements of control or TMPRSS-overexpressing cells incubated with BOC-QAR-AMC for 75 minutes at 37 ˚C. Wells containing PBS and BOC-QAR-AMC were used as background fluorescence reads. **C.** Fluorescence of control or TMPRSS2-overexpressing cells was measured every 15 minutes for a total time of 180 minutes. The average proteolytic activity rate per minute **(D)** or hour **(E)** of control or TMPRSS2-overexpressing cells. Fluorescent signal was measured by the UV filter (excitation 365 nm and emission 410 nm). Data in B, D, and E represent the mean ± SD with interquartile ranges in B and D and individual data points in E.

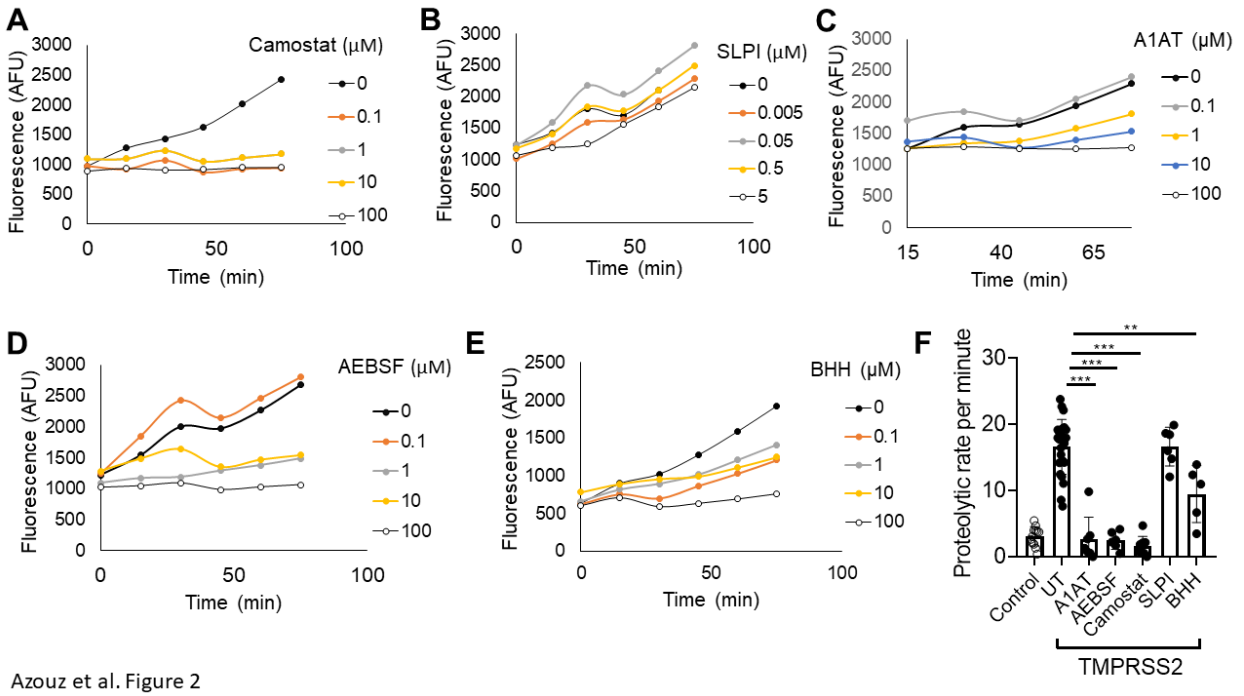

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Figure 2. The effect of protease inhibitors on TMPRSS2 activity. Fluorescence of TMPRSS2-overexpressing cells was measured every 15 minutes in the presence of the indicated concentrations of camostat mesylate **(A)**, SLPI **(B)**, A1AT **(C)**, AEBSF **(D)** or BHH **(E)**. **(F)** The average proteolytic activity rate per minute of control cells or TMPRSS2-overexpressing cells alone or in the presence of 100 μM of either A1AT, AEBSF, camostat mesylate, or BHH or 5 μM of SLPI. ***P value < 0.0001, **P value < 0.01. Fluorescent signal was measured by the UV filter (excitation 365 nm and emission 410 nm).

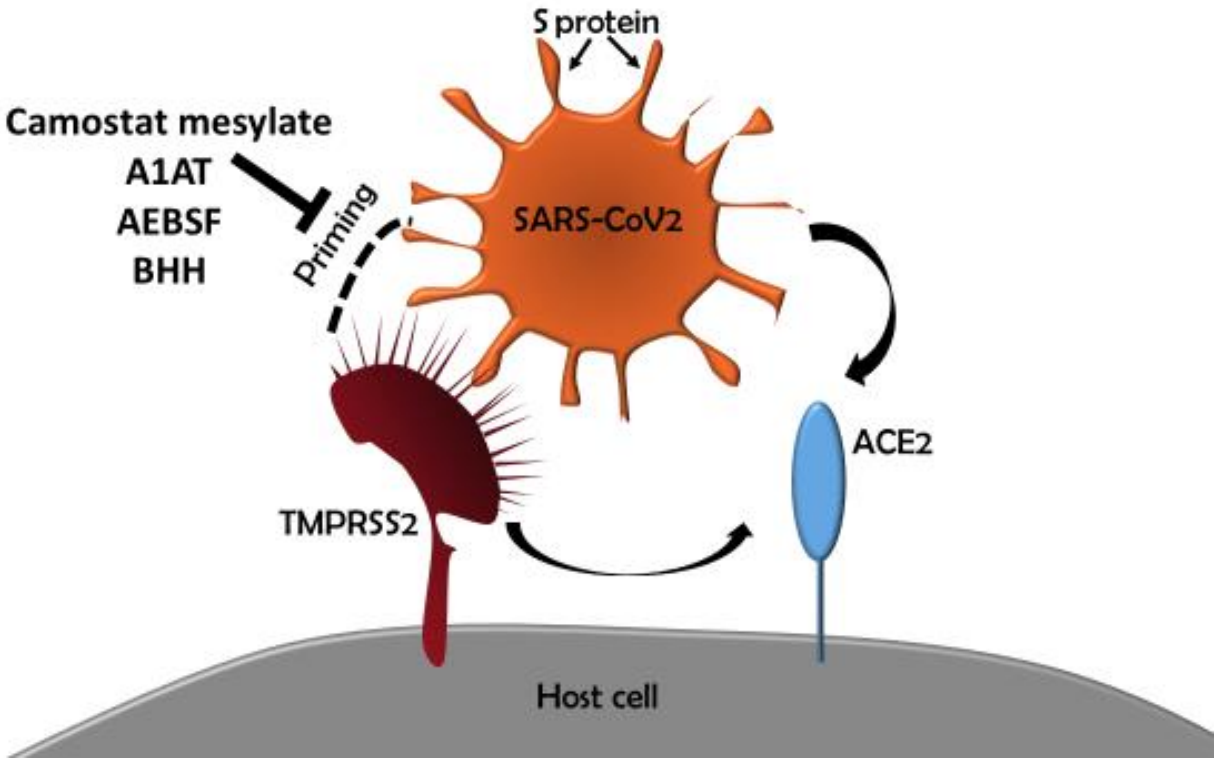

Figure 3. Model of SARS-CoV2 entry and the role of protease inhibitors. TMPRSS2 processes the S protein on the SARS-CoV2 envelope in a process called priming. Priming of the S protein is necessary for binding between the S protein and the host receptor ACE2. TMPRSS2 inhibitors, such as camostat mesylate, A1AT, AEBSF, and BHH prevent the priming of the S protein and therefore block virus entry. In addition, inhibition of TMPRSS2 prevents processing of ACE2, which decreases the infectivity of the coronavirus.