

COVID-19 pandemic; transmembrane protease serine 2 (TMPRSS2) inhibitors as potential therapeutics for SARS-CoV-2 coronavirus.

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The ongoing search for treatments to ease the COVID-19 pandemic concentrates on development of a vaccine or medication to prevent and treat this disease. One of the possibilities is developing new antiviral drugs that are aimed at virus replication or the host factor(s) that are critical to the virus's replication. Serine proteases, which activate the viral spike glycoproteins and facilitate virus-cell membrane fusions for host cell entry, its replication, and spread, are proposed as potential targets for antiviral drug design. Existing literature already provides evidence that transmembrane protease serine 2 (TMPRSS2) may be a promising target. When inhibited it can slow or stop replication of viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for the COVID-19 pandemic. One piece of convincing evidence of the potentially critical role of TMPRSS2 in the coronavirus's replication was provided by an animal study. The replication of influenza viruses was inhibited in TMPRSS2(-/-) knockout mice in comparison to wild type (WT) mice, which experienced a high mortality rate. Existing inhibitors of TMPRSS2 can be divided into two groups. The first include drugs already approved by the FDA or other organizations for treatment of different diseases, including: Camostat (from Japan, produced by Ono Pharmaceutical), aprotinin (Trasylol, produced by Nordic Group Pharmaceuticals) and rimantadine (Flumadine, produced by Forest Pharmaceuticals, Inc.). Existing *in vitro*, *in vivo* and some limited human studies show that this type of drug limits reproduction of coronaviruses and/or prevent the development of viral pneumonia. One study indicated that combined treatment by aprotinin and rimantadine prevented the development of fatal hemorrhagic viral pneumonia, and protected about 75% animals, when the separate administration of aprotinin or rimantadine induced less protection. The second group includes potential drugs not yet approved for the human use, including plasminogen activator inhibitor type 1 (PAI-1) and recently developed small molecular inhibitors. PAI-1 is a serine protease inhibitor that regu-

lates the physiological breakdown of blood clots by inhibiting tissue (tPA) and urokinase (uPA) plasminogen activators. PAI-1 is also an effective inhibitor of various membrane-anchored serine proteases including TMPRSS2. It was reported that PAI-1 inhibited trypsin- and TMPRSS2-mediated cleavage of hemagglutinin and suppressed influenza virus in animals. PAI-1 is human in origin and engineered forms with an extended half-life were developed and could be an attractive addition to the existing TMPRSS2 inhibitors. Finally, derivatives of sulfonlated 3-amindinophenylalanilamide were found to inhibit TMPRSS2 with a high affinity and efficiently block the influenza virus propagation in human cells. This paper is intended to provide a review on possible or hypothetical beneficial effects of (TMPRSS2) inhibitors as one option to fight infection with COVID-19.

COVID-19 | SARS-CoV-2 | TMPRSS2 | inhibitor | therapeutics

There is some confusion regarding nomenclature of the current pandemic, especially among general population. The International Committee on Taxonomy of Viruses selected "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)" as the name of the new virus, and the World Health Organization has started referring to the virus as "the virus responsible for COVID-19 disease" or "the COVID-19 virus" when communicating with the public (1). The recent outbreak of COVID-19 disease worldwide in pandemic proportions can cause a severe acute respiratory condition and has already cost many human lives. This is contrary to the family *Coronaviridae*, in which infections are associated mostly with mild respiratory conditions. The exponential growth of this disease already warrants drastic action in many countries and necessitates an urgent search for possible medications.

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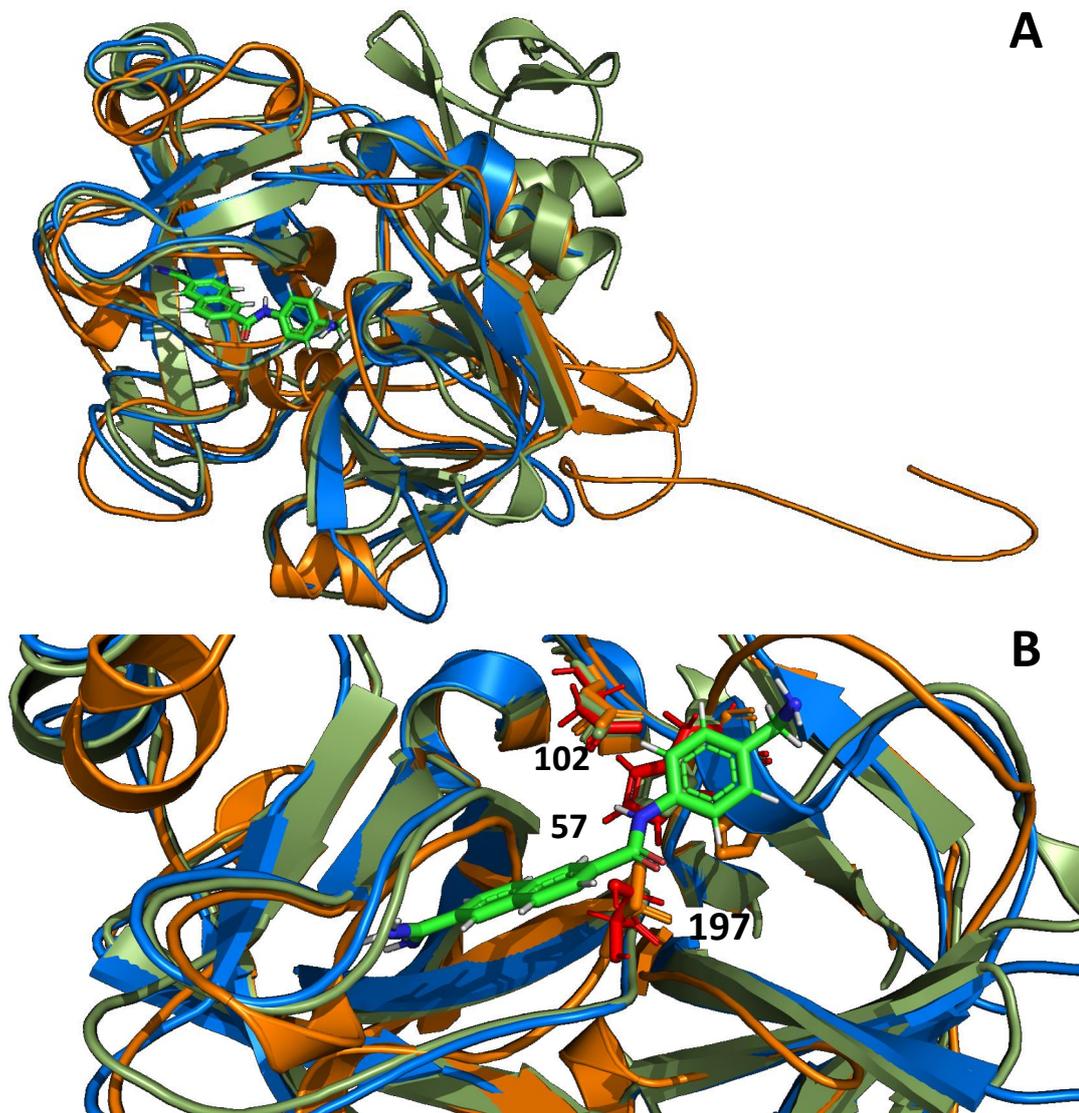


Figure 1. A: TMPRSS2 homology models based on 5ce1 (serine protease hepsin) in brown, 1gpz (zymogen catalytic domain of complement protease C1R) in green, aligned with urokinase 4fuc in blue. Transmembrane domain of two models differ significantly in part to be shorter in 5ce1 and 1gpz than in TMPRSS2. B: enlarged models of TMPRSS2 aligned with uPA. Catalytic triad (57, 102, 197) has very similar spatial positioning with exception of histidine 57 (numbered 296 in the model) of TMPRSS2 modeled after 5ce1. Urokinase inhibitor (6-[(Z)-Amino(imino)methyl]-N-[4-(aminomethyl)phenyl]-4-(pyrimidin-2-ylamino)-2-naphthamide) positioned in the specificity pocket is colored by atoms (carbon in green, nitrogen in blue, oxygen in red, hydrogen in gray), uPA amino acids 57, 102, 195 are shown in red.

Attempts to develop new antiviral drugs are concentrating on elements that aim to impact virus replication or host factor(s) that are critical to viruses replication (2). Existing literature already provides evidence that transmembrane protease serine 2 (TMPRSS2) is one of the promising targets and when inhibited can slow or stop replication of viruses. This paper is intended to provide quick review on possible or hypothetical curable effects of (TMPRSS2) in-

hibitors as one of the options to fight this disease. Cleavage of the viral spike glycoproteins by serine protease causes their activation and facilitates virus-cell membrane fusions leading to host cell entry, replication, and spread. One of the serine proteases essential for viral infectivity is a multidomain type II transmembrane serine protease TMPRSS2 (3). Therefore, TMPRSS2 emerged and was proposed as a potential target for antiviral drug design.

The TMPRSS2 gene is found at human chromosome 21q22.3,112 and encodes a protein of 492 amino acids. TMPRSS2 is a multidomain type II transmembrane serine protease containing two chains: a non-catalytic transmembrane chain formed by amino acids 1 - 255 and a catalytic chain consisting of amino acids 256 - 492. As typical for serine proteases, the active site contains three amino acids of catalytic triad: histidine 296, aspartic acid 345 and serine 441, which in different serine proteases are commonly numbered as histidine 57, aspartate 102, and serine 195 according to the chymotrypsin numbering (4).

No high-resolution structure of the TMPDSS2 is known, only homology models from SWISS-MODEL (5) based on deposited in the Protein Data Bank structures of proteins: serine protease hepsin, 5ce1 and zymogen catalytic domain of complement protease C1R, 1gpz. However these proteins share only 38% identity making the search for inhibitors by molecular modeling methods rather difficult (6-8). Both models produce very similar structures of the catalytic domain but differ significantly in the transmembrane domain. Furthermore, when models are superimposed with X-ray structures of other serine proteases - urokinase (4fuc), both structures and uPA have very similar positions of the catalytic triad (His, Asp, Ser) and similar deep specificity pockets (9, 10). This strongly suggests that existing serine proteases inactivators can provide a pool of potential TMPDSS2 inhibitors (Figure 1).

TMPRSS2 is a member of the hepsin/TMPRSS subfamily, including an additional six proteolytically active enzymes. Unfortunately, the physiological role of this subfamily is still relatively unknown (3). In humans TMPRSS2 is expressed in lungs, prostate and many other tissues, mostly in epithelial cells though the physiological function of TMPRSS2 there is unknown (11). The majority of available literature on TMPRSS2 is related to prostate cancer (12-14), with less focused on viral infections. Nevertheless, it is well established that replication of coronaviruses depends on binding of the viral proteins to cellular receptors followed by cleavage of glycoproteins in their spikes by host cell proteases, including TMPRSS2 (11, 15, 16). Convincing evidence of the potential role of TMPRSS2 in the coronavirus's replication was provided by *Tarnow et al.* (16). They found that H7N9 and H1N1 replication of influenza viruses were inhibited in TMPRSS2(-/-) knockout mice in comparison to WT mice which developed severe disease (100%) with high mortality rates (20%); this was not observed for H3N2 virus (16). This is related to the fact that cleaving hemagglutinin (HA) of H3N2 is facilitated by different serine protease, namely TMPRSS4 (17). These furthermore corroborate the importance of TMPDSS2 or TMPDSS4 inhibition in hopes of developing new antiviral drugs.

Camostat mesylate inhibits TMPRSS2

Camostat produced in Japan by Ono Pharmaceutical is already approved for clinical use for the treatment of cancer and is effective against some viral infections, but it can also inhibit fibrosis, some kidney disease, and pancreatitis (18, 19). Since Camostat is a serine protease inhibitor and serine proteases control many functions in the body, it is no surprise that Camostat has a diverse range of uses and that it is an inhibitor of the TMPRSS2. Inhibition of TMPRSS2 partially blocked infection by SARS-CoV and human coronavirus NL63 in HeLa cells (20). Another in vitro study showed that Camostat significantly reduced the infection of Calu-3 lung cells by SARS-CoV-2, the virus responsible for COVID-19 (15, 20, 21). In their very recent paper, *Hoffmann et al.* concluded that SARS-CoV-2 binds to the angiotensin converting enzyme 2 receptor (ACE2) for entry and proteolysis by TMPRSS2, which is a prerequisite for virus fusion and propagation (15). Moreover, they have found that

an inhibition of TMPRSS2 blocks infection of lung cells and thus such an inhibitor could be potentially used against COVID-19. They also used Camostat mesylate, which is known to be effective against some viral infections (18, 19). *Ikedo et al.* reported observing no serious adverse effects after seven days treatment by Camostat of nephrotic syndrome related to diabetic nephropathy (22). These facts might constitute an immediate treatment option against SARS-CoV-2 infection (15, 18, 19, 23). So far very little is known about side effects when used against COVID-19. Fortunately, they are more potential TMPRSS2 inhibitors that can be immediately used or with quick FDA approval to treat COVID-19.

Aprotinin and rimantadine inhibit TMPRSS2

One such inhibitor is aprotinin, under the trade name of Trasylol, previously produced by Bayer and now by Nordic Group Pharmaceuticals. Aprotinin is a small protein bovine pancreatic trypsin inhibitor (BPTI) used as an antifibrinolytic agent. Trasylol is used as a medication administered by injection to reduce bleeding during complex surgery.

Zhirnov et al. reported that aprotinin and other agents, such as leupeptin (broad cysteine, serine and threonine protease inhibitor) limit the reproduction of human and avian influenza (24). In another paper the authors demonstrated that combined treatment with aprotinin and rimantadine (another antiviral drug under the trade name Flumadine) prevented the development of fatal hemorrhagic viral pneumonia, and protected about 75% animals, when the separate administration of aprotinin or rimantadine induced less protection (35% and 15% respectively). In two separate publications the authors proposed that aprotinin can be delivered as an intrapulmonary aerosol (25, 26). This route seems to be preferred versus intravenous administration since it promises less side effects of aprotinin.

Mangano et al. reported that use of aprotinin was associated with a risk of renal failure, myocardial infarction, heart failure, stroke, or encephalopathy among patients undergoing complex coronary-artery surgery. They described that neither aminocaproic acid nor tranexamic acid used in antifibrinolytic therapy was associated with an increased risk (27). This led to a temporary suspension of Trasylol by the FDA. Contrary to that publication numerous reports describe aprotinin as a safe and superior to aminocaproic acid or tranexamic acid (28-31).

However, after lifting aprotinin suspension, the FDA recommended that: "physicians consider limiting Trasylol use to those situations in which the clinical benefit of reduced blood loss is necessary to medical management and outweighs the potential risks and carefully monitor patients" (32). Flumadine is well-tolerated and is associated with only modest side effects such as nausea, vomiting, loss of appetite, stomach pain (33-35). The use of these two drugs is less publicized than Camostat mesylate but is equally attractive since they are approved by the FDA.

PAI-1 inhibits TMPRSS2

The other option for inhibition of TMPRSS2 is plasminogen activator inhibitor type 1 (PAI-1). PAI-1 in humans is encoded by the SERPINE1 gene and is also known as endothelial plasminogen activator inhibitor or serpin E1 (36, 37). PAI-1 is a serine protease inhibitor with major functions in the regulating physiological breakdown of blood clots by inhibiting tissue plasminogen activator (tPA) and urokinase (uPA) (10, 38, 39). PAI-1 presents a "pseudo-substrate" of its binding loop to the protease, the loop is cleaved and later forms a covalent complex with the protease (11). It is less commonly known that PAI-1 is the effective inhibitor of various

other membrane-anchored serine proteases (11, 36, 38) including TMPRSS2 (15, 40).

PAI-1 is not approved by the FDA as drug, but it is a human protein present in blood and in a variety of tissues. *Dittmann et al.* reported that PAI-1 inhibited trypsin- and TMPRSS2-mediated cleavage of hemagglutinin and suppressed H1N1 influenza virus in animals (40). These results suggest that localized administration of PAI-1 in the respiratory tract could be a new therapeutic approach for the treatment of influenza virus, coronaviruses, or other respiratory viral infections that require host protease-driven maturation (40). Moreover, *Shen et al.* in their paper suggest that intrapulmonary localized administration of PAI-1 could be a new therapeutic approach for the treatment of the influenza virus and other coronaviruses as well (41). They also emphasize the importance of TMPRSS2 protease inhibition. PAI-1 converts itself into a latent inactive form with a half-life of two hours, so if possible, PAI-1 with an extended half-life could be used. Numerous examples of such variants have already been developed, extending the half-life from 6h to over 700h (39, 42).

The side effects of PAI-1 in humans can be difficult to determine since it is not approved to be used as a drug. However, it seems that higher than normal levels of PAI-1 in blood could be tolerated, except during pregnancy. It was reported that women with genetic polymorphisms for plasminogen activator inhibitor-1 4G/5G suffer from recurrent miscarriages (43, 44). This type of polymorphism results in higher PAI-1 levels; when PAI-2 raises during pregnancy in placenta, the combined PAI-1 and PAI-2 inhibitory activity results in the inability of plasmin to lyse blood clots in the placenta (45, 46). PAI-1 does not induce blood clots, rather it prevents lysis by inhibiting uPA or tPA, preventing plasminogen activation to plasmin that is the clot dissolving enzyme (47). Furthermore, animals treated with PAI-1 systemically for two weeks showed no adverse effects (47-49). Nevertheless, safe levels of PAI-1 would have to be established in the future.

Small molecular inhibitors of TMPRSS2

Development of synthetic inhibitors of TMPRSS2 is the other option in the therapy of COVID-19. Historically, these were de-

veloped as anticancer drugs. Numerous inhibitors were tested and some containing 4-amidinobenzylamide yielded compounds with inhibitory potency in the submicromolar range against TMPRSS2(3). An improved potency was discovered for sulfonylated 3-amidinophenylalanamide derivatives which exhibited blockage of influenza virus propagation in airway epithelial cells (3). *Paszti-Gere et al.* described different small molecular inhibitor I-432 of high affinity, that inhibits TMPRSS2 and can be used in coronavirus treatment whenever TMPRSS2 is involved in the spike protein activation (50).

One of the potential problems of these inhibitors is unknown and/or limited selectivity against closely related serine proteases such as: thrombin, uPA, tPA, plasmin, Factor Xa and others. Even if these can be attractive candidates for COVID-19 treatment their specificity against target protein must be confirmed and toxicological studies should be completed before any use in patients.

Conclusion

On the basis of this mini review it seems that TMPRSS2 could be a potential and attractive target to be seriously considered for SARS-CoV-2 antiviral therapy. The most promising candidates for immediate use are inhibitors that are already approved by the FDA or similar agencies abroad for different diseases, which includes: Flumadine, Trasylol and Camostat mesylate. PAI-1 might be an attractive remedy as well since it is a human protein, but requires approval of ethical committees for experimental use and by the FDA in the future. One of the appealing options of using PAI-1 in the therapy is the existence of many PAI-1 mutants with different half-life activities that make possible regulating its activity in broad range (2-700h). Small molecular inhibitors of TMPRSS2 require validation of their specificity against other serine protease and need toxicological studies, therefore their immediate use for COVID-19 patients is unlikely.

Conflict of interest

JJ is a coauthor of patent on plasminogen activator inhibitor with very long half-life (51).

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