REVIEW

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Repurposing and discovery of transmembrane serine protease 2 (TMPRSS2) inhibitors as prophylactic therapies for new coronavirus disease 2019 (COVID-19)

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The global pandemic of COVID-19 disease is caused by the pathogenic factor called SARS-CoV-2. Meanwhile, a series of vaccines and small-molecule drugs, including the mRNA vaccines and Paxlovid®, have been approved, but their efficacy is decreased significantly due to the constant emergence of mutant viral strains. The R&D of host-directed therapeutics has great potential to overcome such limitations and provide new prevention and therapy options for patients with COVID-19 or high-risk group for SARS-CoV-2 infections. Transmembrane serine protease 2 (TMPRSS2) is belonging to a protein family with highly conserved serine protease domain whose crucial role in viral entry is to activate the spike protein of viruses to induce the fusion between host cells and viruses. In this review, we sketch the critical position of TMPRSS2 in the SARS-CoV-2 viral entry and summarize the advanced research and development of TMPRSS2 inhibitors, including repurposed drugs, as a new way to fight COVID-19.

1. Introduction

At the end of 2019, a group of patients with unexplained acute respiratory disease emerged in Wuhan, China, caused by an original betacoronavirus named 2019-nCoV (Huang et al. 2020; Zhu et al. 2020). The present outbreak of pandemic acute pneumonia associated with 2019-nCoV is called COVID-19, and the causative virus has been classified as Severe Acute Respiratory Syndrome Coronavirus 2, renamed SARS-CoV-2 by reason of this virus being the sister clade of human and bat Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (Gorbalenya et al. 2020). The COVID-19 pandemic has caused catastrophic effects for individuals and the health care system and has placed a heavy burden on the economy worldwide. Due to the urgency of this pandemic, the scientists dedicate themselves to research and development of antivirus vaccines and drug repurposing to diminish SARS-CoV-2-associated severe infections (Tian et al. 2021). Antiviral drugs and vaccines are our most effective weapons against emerging viruses, but there are less than 15 marketed antiviral drugs currently available, and there are considerable challenges to develop a high response rate and safe enough vaccine in such a short period of time when we face health emergencies (Adamson et al. 2021). Clinical trials of a vaccine against SARS-CoV-2 have been recruited and conducted around the world in the past few years of pandemic, but the duration of effectiveness of vaccines is hard to exceed six months due to the SARS-CoV-2 variant emerging frequently (Feikin et al. 2022; Sacco et al. 2022).

Due to the limitations of virus-based therapy, another attractive host-based target, namely TMPRSS2 against SARS-CoV-2, has received considerable attention (Adamson et al. 2021). TMPRSS2 plays an essential role in the process of SARS-CoV-2 invades human cells, and it also has been associated with cell entry of SARS-CoV, MERS-CoV, and influenza viruses (Hornich et al. 2021; Kawase et al. 2012; Tomita et al. 2021). Hoffmann and co-workers demonstrated that SARS-CoV-2 cell entry relies on the binding to ACE2 and then priming by TMPRSS2, which led to particular attention on the small molecule drugs targeting TMPRSS2 as great potential candidates for therapeutic and prophylactic of COVID-19 (Bestle et al. 2020; Hoffmann et al. 2020). Currently, the repurposing drugs Camostat and Nafamostat have been the subject of numerous clinical trials worldwide (Wettstein et al. 2022a). Meanwhile, various novel small molecule inhibitors targeting TMPRSS2 have been redesigned and synthesized, Using high-throughput screening and virtual molecular docking studies (Manandhar et al. 2022; Rahman et al. 2020; Vardhan and Sahoo 2022; Wang et al. 2022). In this mini-review, we emphasise the discovery and repurposing of TMPRSS2 inhibitors as SARS-CoV-2 prophylactics and therapeutics.

2. The structure of TMPRSS2 and its role in the viral entry

2.1. Type II transmembrane serine proteases

In 1997, Paoloni-Giacobino and co-workers first discovered that the important member of the Type II subgroup of serine proteases called TMPRSS2, which is composed of 492 amino acids, is distributed to many tissues and organs. It is mainly expressed in the prostate, with a lower level of expression in the colon, liver, kidneys, small intestine, pancreas, salivary gland, stomach, and lung, and the gene that codes for TMPRSS2 is located on human chromosome 21 (Bugge et al. 2009; Lin et al. 1999; Paoloni-Giacobino et al. 1997).

TMPRSS2 is a trypsin-like serine protease that catalyzes the hydrolysis of specific peptide sites and has unique effects in physiological and pathological processes. TMPRSS2 has three main parts (Fig. 1), which are an intracellular domain, a sing-track transmembrane domain, and a proteolytically active ectodomain that is composed of a low-density lipoprotein receptor type-A (LDLR-A), a Class A Scavenger Receptor Cysteine-Rich (SRCR)

domain, and a C-terminal trypsin-like serine peptide (SP) domain with a classical Ser441-His296-Asp345 catalytic complex (Chen et al. 2010). The noncatalytic domain (LDLR-A+SRCR) and catalytic domain (SP) are tethered by a covalent disulfide bond (Cys244-Cys365), and the catalytic domain needs to be activated by autocleavage at the Arg255-lle256 peptide bond before it exerts catalytic proteolytic function at the specific site (Fraser B. J. et al. 2022). But so far, the normal physiological function of TMPRSS2 has not been fully elaborated, the special function of pathological state of TMPRSS2 was mainly investigated in the current research (Kim et al. 2006).

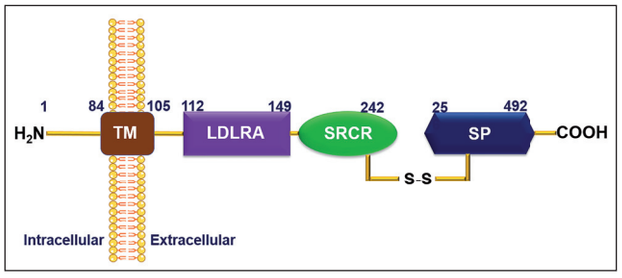

Fig. 1: Structure of TMPRSS2. TM: transmembrane domain; LDLRA: low density lipoprotein receptor class A domain; SRCR: scavenger receptor cysteine-rich domain; SP: C-terminal trypsin-like S1 peptidase domain.

2.2. The role of TMPRSS2 in viral entry

After more than a decade of research with corona and influenza viruses, the mechanism by which viruses invade host cells has been elucidated. The enveloped viruses such as influenza viruses, Middle East Respiratory Syndrome coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) manage to entry by way of a similar pathway in which the glycoproteins of viruses need to be recognized by the host receptors and then catalytically proteolyzed at the specific site of the glycoprotein by a series of proteases including TMPRSS2, furin, and cathepsin L (Böttcher-Friebertshäuser et al. 2011; Gierer et al. 2013; Liu et al. 2020; Yang and Rao 2021).

Shirato et al. (2013) reported that the TMPRSS2 inhibitor Camostat could effectively inhibit MERS-CoV cell entry in the human lung adenocarcinoma cell line Calu-3 but only partially inhibit viral cell entry in Vero-TMPRSS2 cells because it only blocks the TMPRSS2-mediated pathway rather than completely blocking two distinct pathways. Meyer et al (2013) identified that the first synthetic inhibitors of TMPRSS2 potently inhibit influenza virus activation in Calu-3 cells (Meyer et al. 2013). Shen et al. (2020) discovered that influenza A virus propagation can be inhibited by downregulating TMPRSS2 expression by stabilizing the G-quadruplex of TMPRSS2 gene (Shen et al. 2020). Hoffmann et al. (2020) first reported that the SARS-CoV-2 employs the identical cell surface receptor ACE2 as the SARS-CoV for host viral entry, and the TMPRSS2 hydrolyzes the spike protein of SARS-CoV-2 for viral entry (Fig. 2). More importantly, the FDA-approved serine protease inhibitor Camostat mesilate has been proven to block SARS-CoV-2 entry into lung cell lines by inhibiting TMPRSS2 activity (Hoffmann et al. 2020). Matsuyama et al. (2020) identified that the TMPRSS2-expression cells are effortlessly infected by SARS-CoV-2, making the VeroE6/TMPRSS2 cell lines a useful tool for further investigation of the mechanism of viral entry. Meanwhile, Zang et al. (2020) reported that TMPRSS2 and TMPRSS4 facilitate SARS-CoV-2 entry into small human intestinal enteroids. Qiao et al. (2020) demonstrated that androgens could regulate the expression of ACE2 and TMPRSS2, which are strongly correlated with COVID-19 infections. The above finding indicated that TMPRSS2 plays a particular role in viral entry and can promote SARS-CoV-2 infections. Iwata-Yoshikawa et al. (2022) confirmed that TMPRSS2 plays an key role in SARS-CoV-2 invasion because of the Omicron variant infection is significantly reduced in the airway epidermal cells of TMPRSS2-knockout mice model and discovered that SARS-CoV and SARS-CoV-2, including the Omicron variant, enter host cells via a furin/TMPRSS2-mediated signaling pathway in the airways instead of endocytosis.

Based on these knowledge about the important role of TMPRSS2 in the invasion of SARS-CoV-2 into human body, research focused on TMPRSS2, a brand-new target based on the body itself. It is expected that this prophylactic and therapeutic method based on the host itself can be used to meet the unmet needs of patients with SARS-CoV-2 infection.

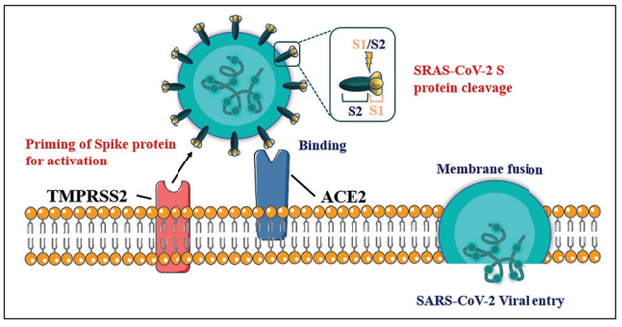

Fig. 2: SARS-CoV-2 viral entry is mediated by ACE2 and TMPRSS2.

3. Repurposing of compounds as TMPRSS2 inhibitors

Drug repurposing is an important field of drug research especially in view of infectious diseases. Older drugs with new indications tend to have higher security, therefore, development for clinical use Is more promising. In this section, we are discussing some older drugs with potential for treatment of COVID-19, which are currently under clinical or preclinical investigation.

3.1. Camostat mesilate and Nafamostat mesilate

Camostat mesylate (Fig. 3) is a phenyl 4-guanidinobenzoate derivative that could inhibit various of proteases including TMPRSS2. Currently, it is marketed in Japan for treating chronic pancreatitis (Mantzourani et al. 2022). In 2015, Zhou et al. identified that SARS-CoV is actuated by the serine protease TMPRSS2 and can be effectively blocked by Camostat in various animal models. Hoffmann et al. (2020) provided an overall assessment of the potential ability of Camostat for blocking of SARS-CoV-2 invasion and the results show that Camostat can potently decrease the Calu-3 cells infection with SARS-CoV-2. It was also discovered that the recently emerged SARS-CoV-2 variants B.1.1.7 and P.1 had escaped neutralizing antibodies, but the TMPRSS2 inhibitor Camostat can robustly inhibit the variant viruses' viral entry (Hoffmann et al. 2021a; Hoffmann et al. 2021b). Esaalmani et al. (2022) reported that combining the furin inhibitor BOS with the TMPRSS2 inhibitor Camostat can completely prevent SARS-CoV-2 infection in Calu-3 cell lines. However, Gunst et al. (2021) reported the results of a COVID-19 clinical trial where 200 mg t.i.d. Camostat mesilate did not show enough efficacy for treating hospitalized patients with SARS-CoV-2 infection. There are still possibilities that Camostat treatment and prophylaxis of SARS-CoV-2 infections are possible in the early stage of the disease (Gunst et al. 2021).

Through rapid hydrolysis of the ester bond, GBPA is emerging as a bioactive metabolite of Camostat. GBPA can also inhibit the invasion of SARS-CoV-2 by blocking TMPRSS2 with less efficiency than Camostat in Calu-3 cells (Beckh et al. 1991; Hoffmann et al. 2021c; Hu et al. 2021; Midgley et al. 1994).

Nafamostat mesylate (Fig. 3) is a potent serine protease inhibitor with naphthalenyl 4-guanidinobenzoate structure initially designed and synthesized by Fujii and co-workers in 1981 (Fujii and Hitomi 1981). It was used for the treatment of acute pancreatitis and cryoglobulimemia by inhibiting a series of serine proteases, including TMPRSS2 (Ueda et al. 2000). In addition, Lu et al. (2016) demonstrated that Nafamostat could inhibit cell proliferation, invasion, and migration by inhibition of the NF-κB and Erk signaling transduction pathways in human colorectal

cancer (CRC) cells; more importantly, a significant antitumor efficacy was observed by the combination of Nafamostat and Oxaliplatin in CRC cells (Lu et al. 2016). Nafamostat has been characterized for its antivirus properties. Yamamoto et al. (2016) first reported that Nafamostat could effectively block MERS-CoV viral entry by inhibiting the membrane fusion of TMPRSS2-mediate employing the protein-based cell membrane fusion assay in the human embryonic kidney cell line 293FT. A series of drugs that can inhibit TMPRSS2, represented by Camostat and Nafamostat, have received constant attention since the outbreak of COVID-19 (Mantzourani et al. 2022; McKee et al. 2020; Wettstein et al. 2022a). Yamamoto et al. (2020) found that the anticoagulant Nafamostat could significantly block SARS-CoV-2 to invade to Calu-3 cell lines with an EC_{50} of 10 nM but inhibit VeroE6/TMPRSS2 cell infection in higher concentrations (EC_{50} around 30 μM), which suggested a cell-type-dependent manner in the SARS-CoV-2 S-protein-mediated cell fusion assay (Yamamoto et al. 2020). A double-blind clinical trial named the RACONA study has been set up to evaluate the practical efficacy of Nafamostat in hospitalized patients with serious COVID-19 (NCT04352400). McFadyen et al. (2020) published a review that elucidated the intrinsic connection between SARS-CoV-2 and thromboembolic complications that are major causes of the high mortality rate in COVID-19. Nafamostat also has antiplatelet effects in addition to blocking TMPRSS2 that have great potential for the treatment of thrombotic complications caused by SARS-CoV-2 infections (Asakura and Ogawa 2020). Si and co-workers developed a microfluidic composite chip that comprises highly differentiated lung endothelial cells, which can simulate viral entry infection and encapsulate human physiology state at a level close to the actual physiological environment of the body, and the group found that the treatment-time window could be doubled by the synergistic effect of Nafamostat with Oseltamivir in an influenza A infection experiment that exhibited prophylactic and therapeutic effects (Si et al. 2021). Ellinger et al. (2021) described a SARS-CoV-2 cytopathicity dataset, which showed that 258 hits, including Nafamostat, can block SARS-CoV-2 cytopathicity by more than 75% when tested in the human colonic adenocarcinoma cell line Caco-2. Krasemann et al. found that SARS-CoV-2 could attack nerve cell lines and engage in active replication across the blood-brain barrier (BBB), resulting in increased interferon signaling in human induced pluripotent stem cells (hiPSC)-derived brain capillary endothelial-like cells (BCECs), and investigated whether the SARS-CoV-2 viral entry nerve cell could be effectively blocked by the TMPRSS2 inhibitor Nafamostat (Krasemann et al. 2022).

3.2. Bromhexine hydrochloride and Gabexate mesilate

Bromhexine hydrochloride is a non-peptide protease inhibitor with the pharmacophore 2,4-dibromo aniline that can potently inhibit TMPRSS2 activity with an IC_{50} of 0.75 μM. It is used as a mucolytic agent in patients with chronic bronchitis, asthma or bronchiectasis, but has also the potential for the prevention of SARS-CoV-2 infection (Chang et al. 2014; Lucas et al. 2014; Maggio and Corsini 2020; Shen et al. 2017). Ambroxol is a metabolite of Bromhexine formed by demethylation and hydroxylation on the cyclohexyl ring, which has the potential to reduce Oxaliplatin-induced neuropathic pain (Furgala-Wojas et al. 2020). Depfenhart et al. (2020) propose using Bromhexine as a repurposing candidate for prophylactic and treatment of COVID-19 in the early stages of the COVID-19 infection. Ansarin et al. (2020) reported the results of an open-label clinical trial study showing that that Bromhexine hydrochloride could significantly decrease the rate of severe acute COVID-19 symptoms, mechanical ventilation, and mortality. However, there were no statistically significant findings in another randomized clinical trial aimed at testing the feasibility of Bromhexine hydrochloride for the treatment of COVID-19 (Li et al. 2020). At the same time, Tolouian and co-workers reported results of another randomized clinical trial where Bromhexine hydrochloride failed to demonstrate sufficient effectiveness for treatment of SARS-CoV-2 infection in a total of 111 hospitalized patients; in particular, the duration of hospitalization could not be reduced by Bromhexine, and the need for mechanical ventilation could not be reduced compared to the standard group (Tolouian et al. 2021). Vila Méndez et al. (2022) disclosed the results of a 28-day multiple-center clinical trial, which showed that Bromhexine could not effectively reduce viral load compared to the group receiving standard of care (SOC). All in all, although Bromhexine hydrochloride can decrease SARS-CoV-2 viral entry *in vivo* experiments, no significant clinical benefit was observed in clinical trials.

Gabexate mesylate is a marketed drug for the treatment of pancreatitis by targeting trypsin-like serine proteases (Cavallini et al. 1996). It has been demonstrated that Gabexate can reduce the viral titers of influenza viruses in cellular level by blocking TMPRSS2 in the host cells (Yamaya et al. 2015). In recent studies, Gabexate mesylate was identified as a TMPRSS2 inhibitor with an IC_{50} of 130 nM but did not reduce SARS-CoV-2 viral entry through S-protein-mediated in lung cell lines (Hu et al. 2021; Shrimp et al. 2020).

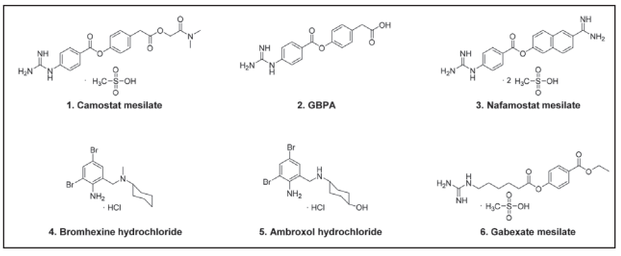

Fig. 3: Structures of Camostat mesilate, GBPA, Nafamostat mesilate, Bromhexine hydrochloride, Ambroxol hydrochloride, and Gabexate mesilate.

3.3. Otamixaban, PCI-27483, and Avoralstat

Otamixaban (Fig. 4) is a specific β-aminoester inhibitor of factor Xa (fXa) that potently inhibits both free and prothrombinase-bound fXa (Guertin et al. 2002), and therefore has considerable potential for the treatment of the acute coronary syndrome. It entered phase 3 clinical trials for the treatment of patients with non-ST segment elevation acute coronary syndrome between April 2010 and February 2013, but the results showed that it did not decrease the ratio of ischemic events compared to a control group but did increase bleeding events (Steg et al. 2013). Rensi et al. (2020) first reported Otamixaban as having great potential for development as an anti-SARS-CoV-2 drug by inhibiting the TMPRSS2 of the host cell in virtual screening. Hu et al. (2021) also reported that Otamixaban is a potent TMPRSS2 inhibitor that can effectively block SARS-CoV-2 viral entry in Calu-3 cell lines by S-Protein particles of SARS-CoV-2 entry assays. Hemple et al. (2021) discovered that Otamixaban has dramatic cooperative effects with Camostat or Nafamostat in Calu-3 cell lines but has lower potency compared with Camostat or Nafamostat when single drug administration is used. The authors consider that the primary target fXa of Otamixaban may have played a special role in the difference between a single dose and a drug combination (Hempel et al. 2021a).

Shrimp et al. (2022) reported and demonstrated a suite assay for the discovery of novel inhibitors of TMPRSS2 for blocking SARS-CoV-2 cell entry, in which initially the specific library compounds were screened by fluorogenic high-throughput screening assay for the evaluation of biochemical activity, then an orthogonal biochemical assay was carried out by mass spectrometry. Finally, the hit compounds from the previous screening were evaluated by a cell-based S-Protein particles of SARS-CoV-2 entry assays. In the above study, PCI-27483 was distinguished between the TMPRSS2 inhibitor and other protease inhibitor (Shrimp et al. 2022). PCI-27483 was primarily synthesized as a powerful and selective serine protease Factor VIIa (FVIIa) inhibitor, constituting a complex with TF (tissue factor) that as an indispensable part in the blood clotting cascade (Morrissey et al. 1997). The relationship between FVIIa/TF and tumor growth and angiogenesis was found in the follow-up study (Hembrough et al. 2003). In 2019, Ramanathan et al. reported the results of a Phase 2 study of PCI-27483,

which showed the PCI-27483-gemcitabine combination did not display better clinical efficacy in pancreatic cancer (Ramanathan et al. 2019). In 2021, Sun and co-workers first characterized PCI-27483 as having the potential to be a candidate for repurposing for the therapy of SARS-CoV-2 viral entry on the strength of a computational tool named 3DPhyloFold and demonstrated that PCI-27483 could effectively block SARS-CoV-2 infection *in vitro* and *in vivo* (Sun et al. 2021).

Cornpropst et al. (2016) reported from a Phase 1 study that Avoralstat (Fig. 4) was tolerated and could be sufficiently inhibit plasma kallikrein, whose overexpression can cause hereditary angioedema (HAE). In another clinical trial, Avoralstat was demonstrated to have enough efficiency for prophylaxis of hereditary angioedema as a first-line oral prophylaxis drug with the mechanism of action on prekallikrein to plasma kallikrein (Aygoren-Pursun et al. 2016). In 2018, Phase 3 hereditary angioedema prophylaxis data was reported that showed Avoralstat did not provide enough protection for angioedema attacks, which was inconsistent with an earlier Phase 2 study that may have been associated with the PK of Avoralstat (Riedl et al. 2018). Sun and co-workers first identified that Avoralstat could not only block SARS-CoV-2 viral entry but reduce viral replication in human airway epithelial cells. In a further *in vivo* experiment for proof of concept, Avoralstat successfully inhibited SARS-CoV-2 infection in a mice model, compared with the similar efficacy of Camostat, which may be a result of its longer plasma half-life (Sun et al. 2021).

3.4. Enzalutamide, Homoharringtonine and Halofuginone

In 2021, Leach et al. identified that the antiandrogen Enzalutamide (Fig. 4) could downregulate TMPRSS2 and reduce the cell entry of SARS-CoV-2 by disturbing the expression of androgen receptors (AR). The group identified that the expression of TMPRSS2 is reduced by Enzalutamide in the human non-small cell lung cancer cell line A549 and mouse lungs, respectively, in addition to proving antiandrogens can reduce SARS-CoV-2 infection successfully (Leach et al. 2021). Otherwise, Deng et al. (2021) reported that the level of ACE2 and TMPRSS2 can be regulated by androgen in mouse and human cells, while Samuel et al. (2020) found that an antiandrogenic drug can reduce ACE2 and SARS-CoV-2 infection by regulating androgen signaling in hESC-derived lung organoids. But contrary to the finding here, Li et al. (2021) found that Enzalutamide significantly reduced SARS-CoV-2 invasion to human prostate cell lines while showing no antiviral activity in mouse epithelial cells and human lung cells. All in all, there are promising applications that interfere with the expression of ACE2 and TMPRSS2 to prevent viral entry through androgen deprivation therapy (ATD), even though the mechanisms that regulate TMPRSS2 and ACE2 expression levels have not been elucidated so far.

In 2021, Chen and colleagues identified drugs that can limit SARS-CoV-2 cellular entry by changing the stability of the TMPRSS2 protein. Homoharringtonine and Halofuginone (Fig. 4) are the two most active lead compounds among the 2560 post-market or current clinical trial compounds. Both drugs were able to markedly increase the tolerance of calu-3 cells to SARS-CoV-2 at a sub-micromolar level. The group further demonstrated that Halofuginone modulates the expression level of TMPRSS2 through the ubiquitin-proteasome system, which includes E3 ubiquitin ligase DDB1- and CUL4-associated 1 (DCAF1) (Chen et al. 2021).

Although the repurposing drugs described above have exhibited superior ability of anti-SARS-CoV-2 invasion in the preclinical studies, the performance of these drugs has been unsatisfactory in clinical trials, to be specific, Bromhexine hydrochloride did not show a significant therapeutic effect on COVID-19 compared with the standard care group. Given the limitations of repurposing drugs, it is crucial to design and synthesize novel inhibitors of TMPRSS2 to block SARS-CoV-2 infecting the human body.

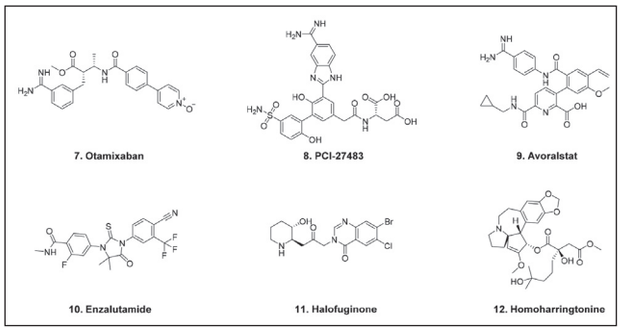

Fig. 4: Structure of Otamixaban, PCI-27483, Avoralstat, Enzalutamide, Halofuginone, and Homoharringtonine.

4. Discovery and identification of novel agents as TMPRSS2 inhibitors

Repurposing marketed drugs clearly does not meet the current need of people for disease treatment, so to acquire more potent therapeutic drugs it is necessary to obtain lead compounds with novel chemical structures for example through high-throughput screening and structure-based design strategies. In this section, novel lead compounds or drugs targeting TMPRSS2 to prophylaxis and treatment SARS-CoV-2 invade humans are summarized.

4.1. NCGC00386945 and BC-11

NCGC00386945 (Fig. 5) was first synthesized as a selective and potent 5-HT_{1D} antagonist by Liao et al (Liao et al. 2000). Hu et al. (2021) reported a new inhibitor named NCGC00386945 that exhibited potent suppression against TMPRSS2 with an IC_{50} of 1.24 μM while also showing 50% inhibition in S-Protein particles of SARS-CoV-2 entry assay in Calu-3 cell lines. NCGC00386945 represents a potentially novel pharmacophore structure that is excellently targeting TMPRSS2.

BC-11 (Fig. 5) is a guanyl-substituted phenylboronic acid derivative originally synthesized as a serine protease inhibitor (Greenidge et al. 2003). Longo et al. (2015) identified BC-11's cytotoxic activity against triple-negative MDA-MB-231 cell lines with an ED_{50} of 117 μM. Moumbock et al. (2023) reported that BC-11 could be used as a viral entry inhibitor by directly binding to and inhibiting the activity of TMPRSS2 with an EC_{50} of 66 μM. Due to its simple chemical structure, it has considerable potential for further structure optimization and modification to obtain more active lead compounds that can specifically target TMPRSS2.

4.2. MM3122, N-0385, Cpd. 5 and Omicsynin B4

In 2021, Mahoney and co-workers discovered a new class of covalent inhibitors that can significantly inhibit SARS-CoV-2 and MERS-CoV cell entry with the ketobenzothiazole (kbt) skeleton, and the most active candidate compound, MM3122 (Fig. 5), inhibiting the enzyme catalytic activity of TMPRSS2 protein with an IC_{50} of 340 pM and blocking viral entry into Calu-3 cell lines with an EC_{50} of 430 pM. Further, MM3122 could also block the cellular entry of MERS-CoV with an EC_{50} of 870 pM (Mahoney et al. 2021).

In 2022, Shapira et al. identified that a new potent peptidomimetic inhibitor, N-0385 (Fig. 5), displayed robust anti-virus efficacy both *in vitro* and *in vivo* by inhibiting the activity of host cell protease TMPRSS2, which can inhibit the viral entry of SARS-CoV-2 mutant strains, for instance B.1.1.7 and B.1.617.2, at low nanomolar concentrations in Calu-3 cell lines. More importantly, N-0385 showed powerful prophylactic and therapeutic effects against SARS-CoV-2 in a newly constructed transgenic mouse model of severe COVID-19 (Shapira et al. 2022). Recently, Cao and co-workers have focused on the mechanism of action of N-0385 inhibiting SARS-CoV-2 to treat COVID-19 through the analysis of virtual molecular dynamics and molecular docking. Preliminary results indicated that N-0385 might act on multiple targets, including TMPRSS2, ACE2, DDP4, and TLR7, to disturb the process of immune recognition and inflammatory-related

response (Cao et al. 2022). Because of this robust research evidence, a class of novel small molecules targeting the protease TMPRSS2 has become a promising strategy to block viral infection in the early stages of COVID-19. Thus, Keller et al. (2022) point out that N-0385 has the potential to be beneficial for people without vaccination or with poor vaccine responses by causing host-directed inhibition of TMPRSS2.

In recent studies, Wettstein et al. developed and identified peptidomimetics inhibitors of TMPRRSS2, and the active compound Cpd. 5 can inhibit SARS-CoV-2 entry with similar efficacy to Camostat mesylate, while the inhibitor Cpd. 5 displayed favorable stability in blood serum and plasma for at least one week (Wettstein et al. 2022b). Li et al. (2023) reported that the natural secondary metabolites of *Streptomyces sp.* 1647, Omicsynin B4 (Fig. 5) , has potent anti-SARS-CoV-2 activity through inhibition of activity of TMPRSS2 and cathepsin L, and can significantly block mutant strains of SARS-CoV-2 invading into host cells in various cell lines (Li et al. 2023). These peptide inhibitors targeting TMPRSS2 represent a series of novel chemical structure candidates for prophylactic and therapeutic antiviral options, and are expected to act as an important emergency tool in potential coronavirus outbreaks in the future.

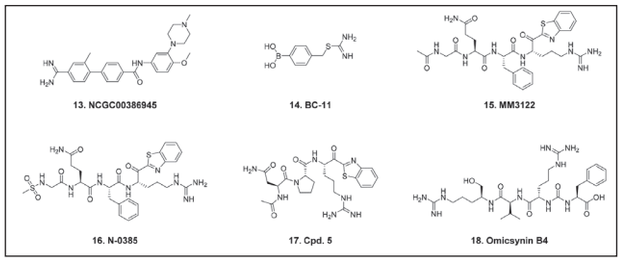

Fig. 5: Structure of NCGC00386945, BC-11, MM3122, N-0385, Cpd. 5 and Omicsynin B4.

5. Virtual molecular docking studies based on the crystal structure of TMPRSS2-Nafamostat complex

Fraser et al. (2021) first reported the crystal structure of the TMPRSS2-Nafamostat complex in Biorxiv and later determined the X-ray crystal structure of the TMPRSS2-Nafamostat complex when it was modified with a catalytic Ser441 residue (Fig. 6). In addition to these findings, they made a great effort to characterize the chemical structural foundation of the mechanism of SARS CoV-2 viral entry and the action of TMPRSS2 inhibitors (Fraser et al. 2022).

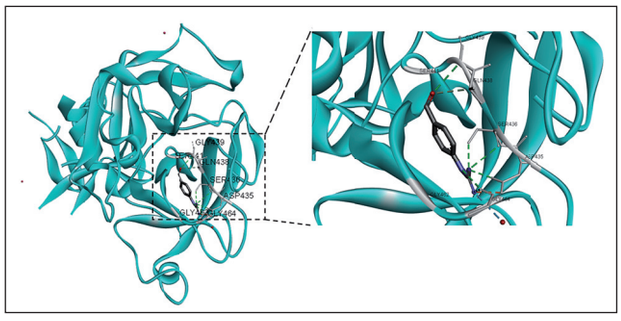

Fig. 6: Crystal structure of TMPRSS2-Nafamostat complex (PDB ID: 7MEQ).

Since the outbreak of COVID-19 worldwide, the broad-spectrum TMPRSS2 inhibitors Camostat and Nafamostat have been receiving continuous attention from the pharmaceutical industry (Hoffmann et al. 2021c; Hoffmann et al. 2020; Sivaraman et al. 2021; Sun et al. 2021; Zhou et al. 2020). Hempel et al. characterized the molecular mechanism of action of the marketed drugs Camostat mesylate and Nafamostat mesylate by combining cell-based biochemical assays with significant molecular docking simulations and Markov modeling (Fig. 7); these data effectively explained that Nafamostat has higher potency towards TMPRSS2 than Camostat (Hempel et al. 2021b). The mechanism of four potential drugs (Camostat, Gabexate, Nafamostat, and Bromhexine) binding with TMPRSS2 was determined by analysis of conformational and energy transduction. The results showed that the guanidinium group of Camostat and Nafamostat is the key motif that undergoes robust interactions with adjacent strong electronegative atoms through the formation of hydrogen bond interaction forces in the molecular docking mode of the drug-TMPRSS2 complex (Zhao et al. 2021). Vankadari et al. (2022) identified the pivotal region of the SARS-CoV-2 S-protein that interacted with the domain of catalytic hydrolysis of TMPRSS2 in the process of TMPRSS2-mediated viral entry by computational and biochemical approaches (Vankadari et al. 2022).

In the current research field of medicinal chemistry, computer-aided drug design and artificial intelligence drug design play a vital role, which can shorten the cycle of new drug research and development and reduce economic costs to a considerable extent. The disclosure of the crystal structure of TMPRSS2-Nafamostat provides a crucial structure basis for rational drug design and development by the above technical means in the future.

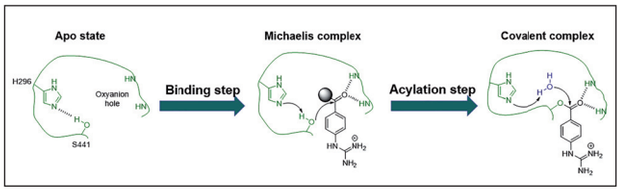

Fig. 7: General mechanism of action by which the pharmacophore groups of Camostat and Nafamostat inhibit TMPRSS2 (Hempel et al. 2021b).

6. Conclusions and perspectives

The COVID-19 outbreak has already infected over 700 million people worldwide, and more than 6 million people died in the last 3 years. Vaccines have been shown to be the most effective weapon against different viruses, including SARS-CoV-2, but not everyone is suitable for vaccination. The current treatment for COVID-19 is divided into two main categories: patient-based symptomatic treatment and SARS-CoV-2-based antivirus therapy. Unfortunately, the above strategies for treatment and prophylactic are limited by the emerging variants of concern. TMPRSS2 is a serine protease that has been demonstrated to play a key role in the SARS-CoV-2 viral entry by activating the spike protein-mediated cell membrane fusion. Camostat and Nafamostat are broad-spectrum serine protease inhibitors that potently inhibit S-protein-mediated viral entry, and they are already in Phase 2/3 clinical trials for COVID-19. Recently, novel TMPRSS2 inhibitors, including MM3122 and N-0385 were synthesized as potential candidate drugs for treatment or prophylaxis of COVID-19. At the same time, the repurposed drugs Otamixaban and Avoralstat that act as TMPRSS2 inhibitors showed potent activity to inhibit SARS-CoV-2 viral entry *in vivo* and *in vitro*. Taken together, it is obvious that TMPRSS2 has a significant capacity as a host-based therapeutic and prophylactic target against SARS-CoV-2 and even other coronavirus infections in the future.

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