

New approaches to influenza therapy

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Influenza and other respiratory diseases are a major cause of global morbidity and mortality. The high mutation rate of influenza viruses renders current vaccines ineffective after a single influenza season and vaccines effective against seasonal influenza do not protect against pandemic influenza. Therefore, antiviral treatment is of central importance to combat influenza. However, currently available antivirals target virus encoded proteins, the ion channel M2 and the neuraminidase, which can change rapidly in the presence of drug, rendering viruses resistant to therapy. As a consequence, new therapeutic approaches must be established and invariable host cell factors, which are required for viral spread but dispensable for cellular survival, are interesting candidates. The activity of host cell proteases which cleave and activate the influenza virus hemagglutinin (HA) is essential for viral infectivity and the responsible proteins are potential targets for antiviral intervention. Recently, several type II transmembrane serine proteases (TTSP) were shown to activate influenza viruses in cell culture and a single TTSP, TMPRSS2, was found to be essential for influenza virus activation in the host. In the present review, we will first provide a brief overview of potential cellular targets for novel anti-influenza therapies. Then we will discuss in detail the influenza virus activation by TTSPs and the therapeutic potential of compounds which block this process.

Keywords: influenza; antivirals; hemagglutinin; type II transmembrane serine proteases

1. Influenza

Influenza and other respiratory diseases are a major cause of global morbidity and mortality. The terms common cold and influenza (flu) are often loosely used to describe any respiratory illness associated with fever and a range of pathogens can be the causative agents [1]. In contrast, authentic influenza is caused by infection with influenza viruses and is characterized by relatively severe symptoms, fever with rapid onset, myalgia, headache, dry cough and nasal discharge [2]. In addition, influenza can be associated with serious complications, most frequently bronchitis and pneumonia caused by secondary bacterial infection. In rare cases, patients may develop myocarditis and encephalitis [3]. Young children, patients with chronic illness and the elderly are at particular risk of developing severe influenza and a fatal outcome is most frequently observed in patients with advanced age, at least in industrialized countries [3]. The virus spreads between humans via aerosols as well as fomites and symptoms develop approximately 18-72 hours after transmission. The highest viral titers are found in nasal and tracheal secretions and patients are infectious up to 7 days after the start of symptoms [2].

Influenza A viruses (FLUAV) cause seasonal epidemics and occasionally pandemics: At least three influenza pandemics were recorded in the last century, the Spanish flu in 1918 (caused by a virus of the H1N1 subtype), the Asian flu in 1957 (caused by an H2N2 virus) and the Hong Kong flu in 1968 (caused by an H3N2 virus) [1]. The Spanish flu had the most dramatic consequences, 30 to 50 million patients died from the disease [4, 5], while the pandemics of 1957 and 1968 were associated with roughly 1 million deaths each [1]. Annual influenza epidemics are responsible for 250.000-500.000 deaths worldwide and wreck massive economic havoc, 87 billion USD in the USA alone [6]. FLUAV infect several avian and mammalian species, with waterfowl being the natural reservoir, and introduction of an animal virus into the human population or the recombination of animal and human viruses are at the root of influenza pandemics [7], as discussed below. In this review, we discuss novel approaches to combat epidemic and pandemic FLUAV, with a strong focus on host cell proteases, which activate FLUAV.

2. Influenza virus life cycle

Influenza viruses belong to the family *Orthomyxoviridae* and are divided into three genera: influenza A, B and C viruses (FLUAV, FLUBV and FLUCV, respectively). FLUAV are additionally divided into subtypes on the basis of the antigenic properties of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA and NA are inserted in the viral membrane (envelope) jointly with the ion-channel M2. The envelope encloses eight segments of genomic RNA, which are associated with the viral RNA polymerase (PB1, PB2 and PA) and coated with nucleoprotein (NP) to form the viral ribonucleoprotein complex (vRNP). The vRNPs are attached to the inner side of the viral envelope via the matrix protein M1 [8].

Infection of target cells commences with binding of HA to receptors on the host cell surface, proteins or lipids modified with α -2,3- or α -2,6-sialic acid (Figure 1, step 1) [9]. Subsequently, virus is taken up into the cell by endocytosis (Figure 1, step 2) and the acidic milieu within endosomes triggers conformational changes in HA, which

drive the fusion of the viral and the endosomal membrane (Figure 1, step 3) [10]. Moreover, M2-mediated transport of protons into the viral interior causes dissociation of the vRNPs from M1 and their release into the cytoplasm of the host cell (Figure 1, step 4) [8]. After transport of the vRNPs into the nucleus, viral mRNAs are synthesized and viral proteins are expressed (Figure 1, steps 5 and 6). The viral surface proteins HA, NA and M2 are imported into the ER and traverse the secretory pathway en route to the cell surface, where HA is proteolytically activated by type II transmembrane serine proteases (TTSPs), as discussed below (Figure 1, step 7) [8]. Newly synthesized genomic RNAs are exported from the nucleus into the cytoplasm and transported to the cell surface (Figure 1, Step 8). At the cellular surface, the RNAs are directed to the site of viral budding by interactions between M1 and the viral surface proteins (Figure 1, step 9), and progeny particles are released [11]. The latter process is promoted by NA-mediated cleavage of sialic acid residues, which prevents binding of new virions to already infected cells (Figure 1, step 10) [11].

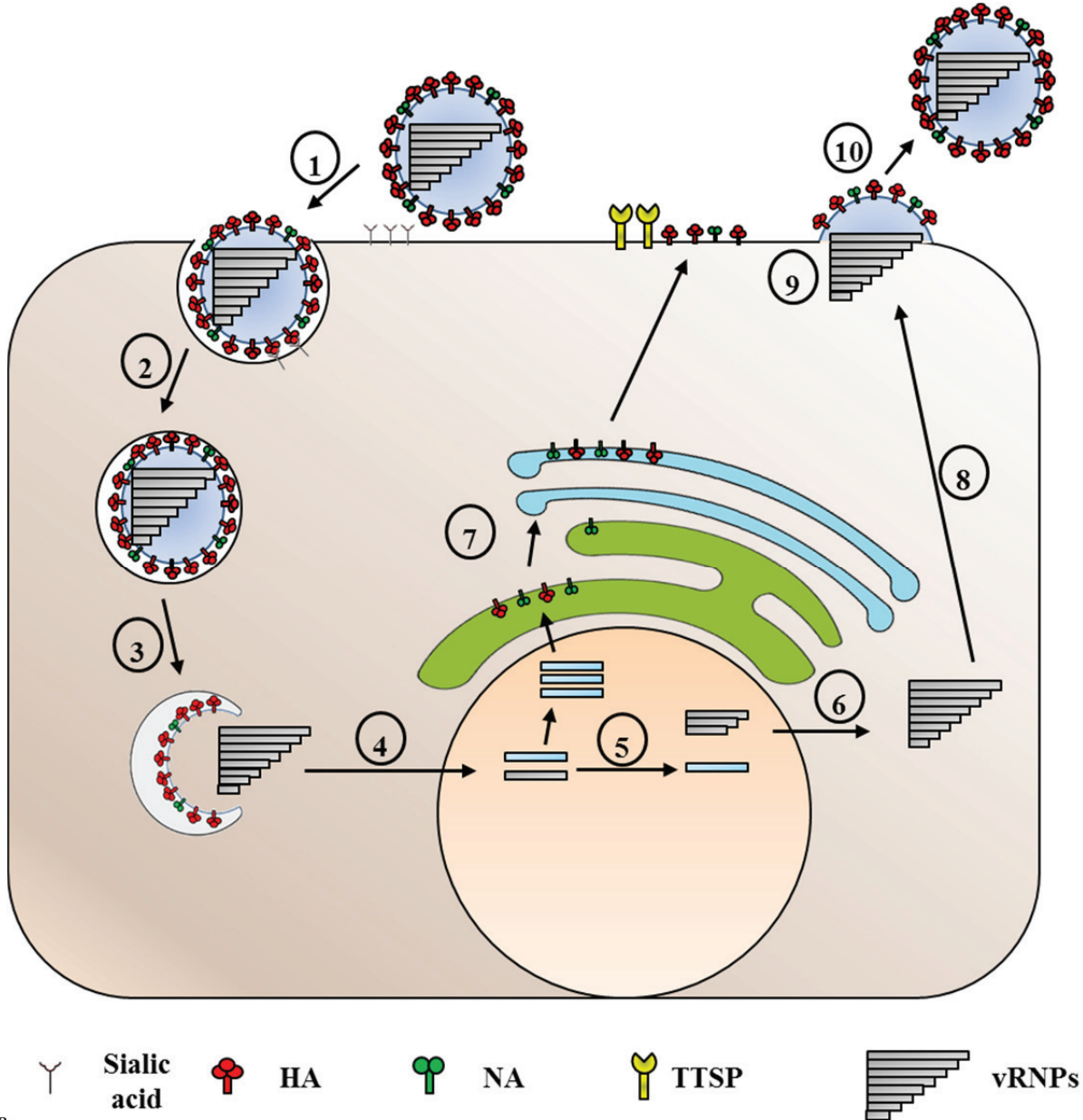


Fig. 1 Influenza virus life cycle.

3. Antigenic drift and shift

The high error rate of the viral polymerase and the segmented nature of the viral genomic information allow for constant viral spread in the human population and for the occasional emergence of pandemic FLUAV, respectively [7]. Thus, antibodies directed against HA are a major immunological defense of humans against infection by FLUAV and subtle changes in the antibody epitopes, due to polymerase errors, allow the virus to constantly evade immune pressure

and to continuously circulate in the human population [12, 13]. The constant change of targets for neutralizing antibodies is termed antigenic drift and is responsible for the annual influenza epidemics (seasonal influenza) [1]. The simultaneous infection of a cell by two different FLUAV allows the interchange (reassortment) of genomic segments and can result in the emergence of viruses with novel antigenic properties, termed antigenic shift [1]. If the human population does not have pre-existing immunity against these viruses, they may spread rapidly and cause an influenza pandemic.

4. Prevention and treatment

4.1 Vaccines

Vaccination induces an adaptive immune response against pathogens and can prevent or at least ameliorate morbidity and mortality associated with infection by many agents, including human papillomavirus, varicella zoster virus, polio virus, measles virus and *Clostridium tetani* [14-17]. In the case of small pox, vaccination even allowed eradication of an infectious disease [1]. Vaccination is also considered to be the most efficient and cost-effective method to prevent influenza and influenza vaccines are available since the 1930s [18]. Currently, trivalent influenza vaccines (TIV) and quadrivalent influenza vaccines (QIV) are used [19]. TIV contain two FLUAV and one FLUBV strain while QIV are composed of two FLUAV and two FLUBV strains, which are either inactivated or live attenuated. The inactivated vaccines comprise either split viruses or viral subunits, i.e. HA and NA. Virosomes, membranes containing HA and NA, have also been used for vaccination [19]. Live-attenuated influenza vaccines are administered intranasally, which mimics the natural route of infection, while inactivated vaccines are applied by needle [19].

Despite their ability to protect against influenza, vaccines have important limitations. The constant antigenic drift of influenza viruses requires that vaccines are reformulated and reapplied on an annual basis [20]. Moreover, vaccines against seasonal influenza will not protect against pandemic influenza, since influenza pandemics are due to the introduction of an antigenically new virus into an immunologically naïve population. Thus, keeping an emerging influenza pandemic in check would require the large scale production and distribution of a new vaccine in short time, which is very challenging [19]. In part, the challenge in producing a vaccine against a pandemic FLUAV is associated with the production system, embryonated hen's eggs. Reverse genetic systems and development of cell culture conditions suitable for large scale production of influenza viruses might help to circumvent these issues [20].

4.2 M2 inhibitors - amantadine and rimantadine

Amantadine was the first identified influenza virus inhibitor and is still in use. Amantadine, as well as the related compound rimantadine, are amine derivatives of adamantane, which block the ion channel M2 of FLUAV but not FLUBV by intercalation into the channel lumen [21]. As a consequence of M2 blockade, the flow of protons into the virion interior is abrogated, which in turn prevents the dissociation of vRNPs and M1 protein and thus the release of the viral genetic information into the host cell cytoplasm [21]. Resistant viruses emerge in up to 50% of the treated patients within days and a single amino acid substitution in the M2 protein is sufficient to confer resistance [22]. In addition, resistance can emerge spontaneously, i.e. in the absence of antiviral pressure. Today more than 90% of the H3N2 strains are resistant, most frequently due to substitution S31M [2]. In addition, virtually all seasonal H1N1 viruses circulating after the 2009 pandemic were found to be adamantane-resistant by the WHO Collaborating Centers [1]. Therefore, amantadine and rimantadine are not recommended in several countries for treatment of influenza caused by FLUAV currently spreading in the human population.

4.3 Neuraminidase inhibitors - zanamivir and oseltamivir

The NA protein promotes release of progeny virions from infected cells by removing sialic acids from factors displayed at the cell surface, including the viral HA [8]. Inhibition of NA prevents virion release and causes aggregation of virions, thereby blocking viral spread [8]. The solution of the crystal structure of NA in complex with ligand suggested that structural analogues of sialic acid could exert potent inhibitory activity. Indeed, two sialic acid analogues, zanamivir and oseltamivir are now widely used for influenza therapy and are active against both FLUAV and FLUBV [23]. A third NA inhibitor, peramivir, was FDA approved in 2014. These drugs can reduce the duration of uncomplicated influenza and can decrease the severity of symptoms, if taken within two days after the appearance of symptoms. Additionally, NA inhibitors may reduce the frequency of serious complications in high risk groups [24].

Oseltamivir resistance requires acquisition of two classes of mutations: First, substitutions that allow enzymatic activity of NA despite the presence of drug, most frequently H274Y [25]. Second, mutations which increase NA expression and activity and thereby rescue the virus from fitness defects associated with the resistance mutation. Oseltamivir resistant viruses seem to arise most frequently in immunocompromised patients but were also observed in the absence of drug. Zanamivir and oseltamivir use different strategies to block the catalytic center of NA and oseltamivir resistance mutations usually do not provide the virus with cross resistance against zanamivir [26]. Indeed,

resistance against zanamivir is infrequently observed. However, it is conceivable that zanamivir resistant viruses emerge and spread in the future and novel therapeutic options to combat such viruses are needed [7, 13].

5. New approaches to influenza therapy

Antivirals currently used for treatment of influenza target viral proteins. However, these proteins are highly variable and adapt rapidly to antiviral pressure, resulting in the emergence of drug resistant viruses, as discussed above. Moreover, spontaneous emergence of mutations conveying resistance to NA and M2 has been documented [27-29]. Finally, inhibitors targeting viral proteins might exert activity against some influenza viruses but not others, as underlined by M2 inhibitors, which are active against FLUAV but not FLUBV. Several new approaches to influenza therapy address these limitations by targeting host cell encoded factors, which are essential for viral spread but dispensable for cellular survival [26]. This strategy has two advantages: Inhibition of host cell factors, which are genetically stable and cannot change in response to drug pressure, might suppress the emergence of resistance. In addition, targeting cellular factors used by diverse viruses for spread might allow the generation of inhibitors with broad antiviral activity. On the other hand, interfering with cellular functions might result in substantial unwanted side effects and this concern needs to be carefully addressed.

Strategies targeting host cell factors essential for virus entry have already been implemented. The use of sialidase (e.g. DAS181 (Fludase), a recombinant fusion protein composed of a sialidase catalytic domain derived from *Actinomyces viscosus* and a cell-surface anchoring domain of human amphiregulin), can protect against infection with avian FLUAV of the H5N1 subtype as well as pandemic 2009 swine influenza virus [30]. Moreover, previously known drugs like clarithromycin, a macrolide used for treatment of tonsillitis and pharyngitis, reduces expression of sialic acid receptors and has thus potential as an anti-influenza drug [31]. After binding to sialic acids, the virus must be transported into late endosomes, where the acidic milieu triggers conformational changes in HA required for fusion of the viral membrane with the endosomal membrane. Therefore, inhibition of endosomal acidification is another option to block virus entry. The antibacterial and antimalarial agents, bafilomycin and concanamycin, elevate the endosomal pH and thus show anti-influenza potential [32]. These macrolids block the vacuolar proton translocating ATPase, which is required for endosomal acidification [33].

The above described strategies target two well-known cellular prerequisites for FLUAV entry, expression of sialic acids and endosomal acidification. Recent genome-wide RNAi screens have defined a multitude of additional factors required for FLUAV spread, many of which may constitute potential drug targets [34-40]. The recent demonstration that knock-down of cathepsin W expression blocks FLUAV entry at the stage of membrane fusion confirms that factors identified in siRNA screens indeed hold potential as targets for antiviral intervention [41].

5.1 HA cleavage by type II transmembrane serine proteases as a new target to influenza therapy

A class of host cell factors essential for influenza virus spread has been known for more than 40 years: Host cell proteases, which process the viral HA are essential for influenza virus infectivity and constitute potential targets for antiviral intervention. Thus, HA is synthesized as an inactive precursor, HA0, and depends on proteolytic cleavage into the surface unit, HA1, and the transmembrane unit, HA2, to transit into an active form which is able to fuse the viral envelope with the endosomal membrane [42]. The activity of many viral glycoproteins depends on cleavage by host cell proteases [43] and this process is called priming or, in the context of influenza virus, activation. Activation is essential for these proteins to be responsive to a subsequent trigger of the membrane fusion reaction, typically receptor binding or, in the case of influenza viruses, low pH.

Historically, the first identified activator of human FLUAV HA was trypsin [44]. Subsequently, other soluble, trypsin-like proteases, including Clara cell tryptase, mini-plasmin and kallikrein were shown to cleave and activate FLUAV HA, suggesting that HA is activated upon release of progeny virions into the extracellular space [45, 46]. However, work by Zhirnov and colleagues [47] demonstrated that cell-associated serine proteases activate FLUAV in cultured respiratory epithelium, and work by Böttcher and coworkers showed that TTSPs, TMPRSS2 and HAT, activate HA upon engineered expression in cell culture [48]. More recently, it was demonstrated that also TMPRSS4, matriptase, DESC1 and MSPL can cleave and activate HA in cell culture [49-54], and that endogenous TMPRSS2 expression is responsible for trypsin-independent spread of FLUAV in Caco2 and Calu3 cells [55, 56]. Moreover, TMPRSS2 was found to be coexpressed with the major receptor determinant of human FLUAV, α -2,6-linked sialic acid, in most parts of the human aerodigestive tract [57]. In 2013, Hatesuer and colleagues demonstrated that FLUAV depends on TMPRSS2 expression for activation in mice. Thus, tmprss2 knock-out mice were shown to be protected from disease upon H1N1 FLUAV infection, due to the lack of robust HA proteolytic processing and ensuing inefficient viral spread [58]. These findings were confirmed by two independent studies [59, 60] and, jointly with the observation that TMPRSS2 is dispensable for normal development and homeostasis [61], define TMPRSS2 as an attractive target for antiviral intervention.

The TTSP family contains at least 20 members, which are subdivided into four subfamilies (TMPRSS/Hepsin, Matriptase, HAT/DESC and Corin) [62]. The proteases of the TTSP family exhibit

a comparable domain organization: The N-terminus is located in the cytoplasm and is followed by a transmembrane domain, a central stem region containing domains potentially involved in protein-protein interactions and a C-terminal serine protease domain [63]. TTSPs are synthesized as inactive single chain proenzymes, zymogens, and undergo autocatalytic activation, i.e. cleavage after a basic amino acid residue in a C-terminal activation domain. After cleavage the protease domain remains associated with the remainder of the protein due to a disulfide bond between stem and protease domain. However, the disulfide bond can be severed, resulting in release of the protease domain in the extracellular space [62, 64]. Although expression levels differ widely, mRNAs for many TTSPs have been detected in the respiratory tract [62-65]. Dysregulated expression of TTSPs has been associated with several cancers, suggesting that blockade of specific TTSPs might be associated with clinical benefits in patients suffering from certain cancers [66, 67].

Despite the key role of TMPRSS2 in FLUAV spread and pathogenesis, at least in mice, no TMPRSS2-specific inhibitors are available, although a set of synthetic inhibitors targeting TMPRSS2 and inhibiting FLUAV spread in cell culture has been described [68]. Nevertheless, protease inhibitors with relatively broad specificity were shown to block FLUAV infection. Aprotinin is a single chain, low molecular weight, globular polypeptide isolated from the bovine lung, which inhibits a wide range of proteases, including human plasmin, trypsin, chymotrypsin, kallikrein, TMPRSS2 and HAT [69]. In cell culture, aprotinin inhibits spread of diverse FLUAV [47, 70]. Moreover, aprotinin application via the respiratory tract was well tolerated and blocked FLUAV spread and pathogenesis in experimentally infected animals [69]. The promising data obtained in animal models warranted testing of this compound as an anti-influenza drug in humans. It was shown that aprotinin can reduce symptoms and shorten the time of illness and the compound was approved as an anti-influenza drug in Russia [69].

Camostat mesylate is a low molecular weight inhibitor of serine proteases used for treatment of acute pancreatitis [71]. Camostat also displays robust anti-FLUAV activity [72, 73] and was shown to block TMPRSS2 [74]. Whether camostat also blocks other members of the TMPRSS/Hepsin subfamily is unclear. Analysis of HA cleavage in transfected 293T cells revealed that TMPRSS4 expression results in cleavage of the HA precursor, HA0, into the HA1 and HA2 subunits, as expected, and that this process is inhibited by camostat (Fig. 2A). Thus, neither HA1 nor HA2 were detectable in cells treated with camostat while the intensity of the HA0 precursor increased (Fig. 2A). These results suggest that camostat can prevent HA activation by TTSPs other than TMPRSS2. It was also tested whether camostat blocks FLUAV spread in Caco2 cells, which were previously found to support FLUAV spread in a TMPRSS2-dependent, trypsin-independent fashion [55]. Infection of 293T cells was examined in parallel, since these cells do not express a HA-activating protease and therefore served as negative control [51]. For this, both cell lines were infected with FLUAV encoding *Gaussia luciferase* [75] and treated with increasing amounts of camostat. Luciferase activity in culture supernatants was measured at 48 h post infection as an indicator for the efficiency of FLUAV spread in the infected cells. Camostat treatment inhibited FLUAV spread in Caco2 cells in a dose-dependent manner but had no effect on the residual viral replication in 293T cells. Finally, camostat treatment was recently shown to protect rodents from lethal infection by SARS-coronavirus [76], which can also be activated by TMPRSS2 [77-79]. These observations indicate that camostat and related inhibitors have potential as antivirals.

Recent work provided evidence that also endogenously produced protease inhibitors might function as defenses against FLUAV. Dittmann and colleagues demonstrated that plasminogen activator inhibitor 1 (PAI-1), the product of the *serpine1* gene, can inhibit the proteolytic processing of HA by TMPRSS2 and HAT [80]. Additionally, they showed that FLUAV infection in *serpine1*^{-/-} mice is more severe compared to wt mice, although the effects observed were modest. Moreover, a separate study showed that hepatocyte growth factor activator inhibitor 2 (HAI-2) can block HA activation in cell culture and can protect mice from influenza [81]. Whether other endogenous protease inhibitors also block HA activation remains to be examined.

6. Conclusions

Currently available antivirals directed against FLUAV target viral proteins, which can rapidly adapt to drug pressure. The frequent emergence of resistant viruses is the consequence. Targeting host cell-encoded and thus genetically stable factors required for FLUAV spread is an alternative approach to antiviral intervention, which may largely suppress resistance development. Host cell proteases which activate HA are attractive targets and protease inhibitors with broad specificity display anti-FLUAV activity. However, potential unwanted side effects are a concern. The recent identification of TMPRSS2 as the central activator of FLUAV *in vivo* indicated that inhibitors specific for this protease might exhibit potent antiviral activity. Importantly, TMPRSS2 specific inhibitors might not be plagued by substantial unwanted side effects since TMPRSS2 knock-out is compatible with normal development and homeostasis in mice. Moreover, the recent finding that diverse respiratory viruses hijack TMPRSS2 for their activation, including MERS-coronavirus and human metapneumovirus [82-85], indicate that TMPRSS2 inhibitors might exert broad antiviral activity.

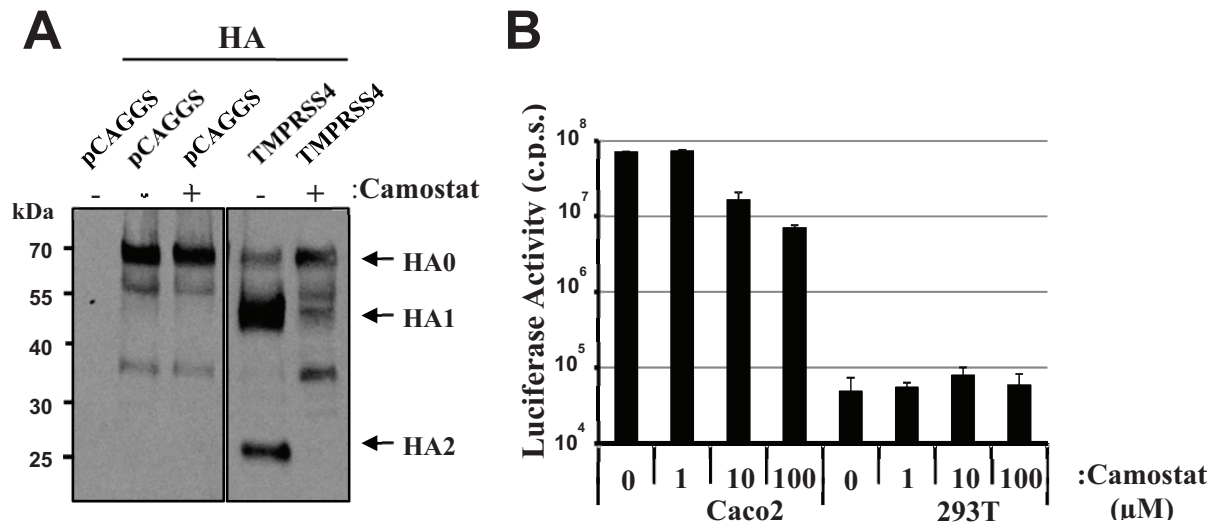


Fig. 2 Inhibition of HA cleavage by camostat. A) 293T cells were cotransfected with plasmids encoding FLUAV HA and TMPRSS4 or empty plasmid (pCAGGS) and treated with camostat mesylate (100 μM). At 48h post transfection the HA cleavage was analyzed by Western blotting. The HA0 precursor (upper arrow) and the HA1 (middle arrow) and HA2 (lower arrow) subunits are indicated. Non-relevant lanes were cut out. B) Caco2 and 293T cells were infected with 100 ffu FLUAV A/PR/8/34 encoding Gaussia luciferase and treated with increasing amount of camostat mesylate. At 48h post infection, the luciferase activities in culture supernatants were determined. The results of a representative experiment performed with triplicate samples are shown. Similar results were obtained in three independent experiments. Error bars indicate standard deviations (SD)

Acknowledgements This work was supported by the Göttingen Graduate School for Neurosciences, Biophysics and Molecular Biosciences (DFG grant GSC 226/1 and DFG grant GSC 226/2), the Leibniz Graduate School Emerging Infectious Diseases and DFG (PO 716/6-1).

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