

Serine Proteases and their Inhibitors in Human Airway Epithelial Cells: Effects on Influenza Virus Replication and Airway Inflammation

Mutsuo Yamaya^{1*}, Yoshitaka Shimotai², Yukimasa Hatachi³, Morio Homma⁴ and Hidekazu Nishimura⁴

¹Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, Sendai, 980-8575, Japan

²Department of Infectious Diseases, Yamagata University Faculty of Medicine, Yamagata, 990-9585, Japan

³Division of Oncology, Kobe City Medical Center General Hospital, Kobe, 650-0047, Japan

⁴Virus Research Center, Clinical Research Division, Sendai Medical Center, Sendai, 983-8520, Japan

*Corresponding author: Mutsuo Yamaya, Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, 2-1, Seiryomachi, Aoba-ku, Sendai, 980-8575, Japan, Tel: 81-22-717-7184; Fax: 81-22-717-7576; E-mail: myamaya@med.tohoku.ac.jp

Received date: February 08, 2016; Accepted date: March 04, 2016; Published date: March 11, 2016

Copyright: © 2016 Yamaya M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Influenza virus replication and the production of inflammatory cytokines are associated with symptoms, including fever, and exacerbation of bronchial asthma and chronic obstructive pulmonary disease. Proteolytic activation of influenza viruses by serine proteases that are produced by airway epithelial cells is essential for viral entry and replication. Transmembrane protease serine S1 member (TMPRSS) 2, TMPRSS4 and TMPRSS11D have been detected certain cells, including the human alveolar epithelial cell line A549 and the surface epithelial cells of the human nasal mucosa, the trachea, the distal airways, and the lung. Several protease inhibitors, including aprotinin, reduce influenza virus replication. We previously demonstrated the following: (1) TMPRSSs (TMPRSS2, 4, and 11D) are present in primary cultures of human tracheal epithelial cells; (2) serine protease inhibitors, such as camostat and aprotinin, reduce the influenza virus replication and the release of the cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)- α into cell supernatants; and (3) camostat reduces the cleavage of an influenza virus precursor protein, HA0, into the subunit HA1. These findings suggest that serine proteases expressed by human tracheal epithelial cells induce the proteolytic activation of influenza viruses and that serine protease inhibitors may reduce viral replication and the resultant production of inflammatory cytokines. Thus, serine protease inhibitors are potential candidates for anti-influenza virus drugs.

Here, we review the expression of serine proteases, the role of serine proteases in influenza virus activation, and the effects of serine protease inhibitors. In this review, we aim to introduce the effects of serine proteases and their inhibitors on influenza virus infection of human airway epithelial cells by discussing the findings of previous studies performed by our group and other research groups. Furthermore, the clinical features and virulence of influenza virus infection are reviewed to clarify the association of virus replication and cytokine release with disease severity.

Keywords: Airway epithelial cell; Camostat; Cytokine; Influenza virus; Proteases transmembrane protease serine S1 member; Serine protease inhibitor

Introduction

Homma and Ohuchi were the first to report that Sendai virus is activated when trypsin cleaves the viral surface glycoprotein and that the cleaved protein fuses the viral envelope to the host cell membrane and causes viral entry and replication [1,2]. Subsequent reports demonstrated that trypsin and other host proteases, by cleaving the surface glycoproteins important for membrane fusion, contribute to the spread of infection and the pathogenicity of many types of viruses, including paramyxoviruses and influenza viruses [3,4].

Serine proteases such as trypsin, transmembrane protease serine S1 member (TMPRSS) 2, TMPRSS4, and human trypsin-like protease (HAT; also known as TMPRSS11D) are important examples of host proteases that are responsible for the proteolytic cleavage of influenza virus hemagglutinin (HA), which is essential for viral genome entry to the cell and for the start of viral replication [5,6]. Furthermore, several protease inhibitors have been demonstrated to reduce the replication

of influenza viruses [7-10], suggesting that these inhibitors may become candidates for anti-influenza drugs.

We previously demonstrated that serine proteases were produced by primary cultures of human tracheal epithelial cells (that retain the function of their tissue of origin [11]) and that proteases activated influenza virus HA through proteolytic cleavage. We also demonstrated that serine protease inhibitors, including camostat, reduced viral replication and inflammatory cytokine release from the cells [12].

Herein, we review the expression of serine proteases in airway epithelial cells, the role of serine proteases in influenza virus activation, and the effects of serine protease inhibitors on viral replication and cytokine release. Clinical features and treatment of influenza virus infection are also reviewed.

Clinical Features of Influenza Virus Infection and Anti-influenza Drugs

Human influenza virus infection causes a rapid onset of constitutional symptoms, including fever and lower respiratory tract symptoms. Chronic obstructive pulmonary disease (COPD) is one of

the leading causes of death worldwide, and the number of patients is increasing among older adults [13,14]. It is generally accepted that cigarette smoke is the most common identifiable risk factor for COPD [15]. Because smoking history is associated with the development of COPD, elderly people are more susceptible to COPD [14,15]. Influenza virus infection can exacerbate bronchial asthma and COPD during winter [16,17]. Therefore, vaccination is recommended to prevent exacerbation of bronchial asthma and COPD and to reduce the mortality rate of older people with COPD [18]. Furthermore, influenza vaccination is recommended for children and non-elderly adults to prevent severe illness in patients with influenza virus infection [19,20].

In addition, anti-influenza drugs, which include neuraminidase inhibitors such as Oseltamivir and Zanamivir, are beneficial in cases of uncomplicated pandemic and seasonal human influenza infection [21-23]. In contrast, certain types of patients with pandemic and seasonal influenza virus infection, including immuno-compromised patients, have died of pneumonia and multi-organ system failure despite intensive drug treatments, which include taking neuraminidase inhibitors and steroids [24,25]. Oseltamivir-resistant influenza A (H1N1) virus infection has been reported, and this type of seasonal influenza has caused severe disease in immuno-compromised patients [26]. In a previous study, Oseltamivir did not reduce inflammatory cytokine production by airway epithelial cells after infection with seasonal influenza viruses with an Oseltamivir-resistant genotype [27]. Moreover, Oseltamivir did not reduce viral titers or viral RNA levels. The 50% inhibitory concentration (IC₅₀) of Oseltamivir for neuraminidase activity in the Oseltamivir-resistant seasonal virus was 300-fold higher than that observed for the pandemic influenza virus [27]. According to a report by Shobugawa et al., oral intake of Oseltamivir is feasible for children younger than 5 years old, whereas Zanamivir inhalation is prescribed for children ≥ 5 years old [28]. However, inhalation of Zanamivir may be difficult for patients with severe infections and for small children. Therefore, alternative anti-influenza virus drugs are needed.

The Virulence of Influenza Viruses

Because influenza viruses with a high virulence can cause severe illness and even fatality, precise information regarding the pathogenicity of these viruses is needed for effective treatment and to prevent confusion and fear during pandemic infection. The pathogenicity of the influenza type A (H5N1) virus has been reported to be associated with the non-structural gene segment [29], which contributes to an increase in inflammatory cytokine production. Furthermore, several studies have reported mechanisms of high pathogenicity, which include an elevation in pulmonary concentrations of inflammatory cytokines (e.g., interleukin (IL)-1, IL-6 and interferon (IFN)- γ), a decrease in anti-inflammatory cytokine production, and an elevation in viral replication [30,31].

Influenza viral replication induces cytokine production and cell damage in the airway epithelium, which is the first target of infection. Infection-induced production of inflammatory cytokines, including IL-6 and tumor necrosis factor (TNF)- α , and proteases may cause damage to airway and alveolar epithelial cells and to vascular endothelial cells [32-34]. This damage may subsequently exacerbate bronchial asthma and COPD and develop into pneumonia and acute respiratory distress syndrome (ARDS) [24,35-37] (Figure 1). IL-6 and TNF- α are associated with cell death and the activation of caspases in swine macrophages after pandemic A/H1N1 viral infection [38]. Patients infected with highly pathogenic influenza viruses experience

increased viral replication and subsequent hypercytokinemia [30,39]. Influenza viral infection-induced cell damage is partly related to the magnitude of viral replication, as well as NF- κ B-p65-mediated IL-6 production and caspase-3 activation [40]. Furthermore, a large number of CD8⁺ T cells and granzyme B⁺ cells have been found to be expressed in autopsy lung tissue specimens from patients infected with pandemic A/H1N1 influenza virus [32]. Granzymes are granule-stored lymphocyte serine proteases, and CD8⁺ cells exert their cytotoxic functions by releasing granzymes in response to influenza virus infection [41]. Because IL-6 induces granzyme production [42], hypercytokinemia during influenza virus infection may activate CD8⁺ cells, resulting in lung damage through the cytotoxic activities of granzymes (Figure 1).

To ameliorate the symptoms associated with the exacerbation of bronchial asthma and COPD caused by virus infection, including influenza virus infection, oral steroids and inhalation of β 2 agonists have been used [43-45]. Furthermore, treatment with antibiotics is recommended when COPD patients have purulent sputum caused by a secondary bacterial infection [46].

Serine Proteases and Their Roles in Influenza Virus Replication in the Airways

The pathway of influenza virus attachment, internalization and uncoating

The hemagglutinin (HA) of human seasonal and classical H1N1 swine influenza viruses binds to the receptor SA α 2, 6Gal, and the HA of most avian and equine viruses binds to SA α 2, 3Gal [47]. Expression of SA α 2, 6Gal was observed in epithelial cells in the nasal mucosa, pharynx, tracheae and bronchi [48-51]. SA α 2, 3Gal is expressed in the human tracheae, nonciliated cuboidal bronchiolar cells and type II cells lining the alveolar wall [49,52].

After the attachment of influenza virus HA to the receptor, viruses enter airway epithelial cells and are internalized by endocytic compartments via four internalization mechanisms: clathrin-coated pits; caveolae; a nonclathrin, noncaveola pathway; and macropinocytosis [53]. Of these mechanisms, the nonclathrin, noncaveola-mediated internalization pathway depends on a low pH, which triggers the fusion of the viral and endosomal membranes [54]. After binding and internalization of the virus by receptor-mediated endocytosis, the activated influenza virus HA, which is cleaved by serine proteases, induces fusion of the viral envelope to the host cell membrane in acidic endosomes [3,4]. The virus then releases its ribonucleoproteins containing viral RNA into the cytoplasm, resulting in the next steps of viral replication [53,55].

The expression of serine proteases in the airways and other organs

TMPRSSs are expressed in a broad range of human tissues (Table 1). TMPRSS2 is expressed in the human nasal and tracheal mucosa, distal airways, and lung [6,12,56,57]. TMPRSS2 is also expressed in the human Caco-2 colon cancer cell line, the human Huh7 hepatocellular carcinoma cell line [58], and swine airway epithelia [59]. TMPRSS4 is expressed in lung cancer tissue [60], the mouse lung [61] and the human trachea [12], and TMPRSS11D is expressed in human bronchi and tracheae [12,62,63] and in swine airway epithelia [59].

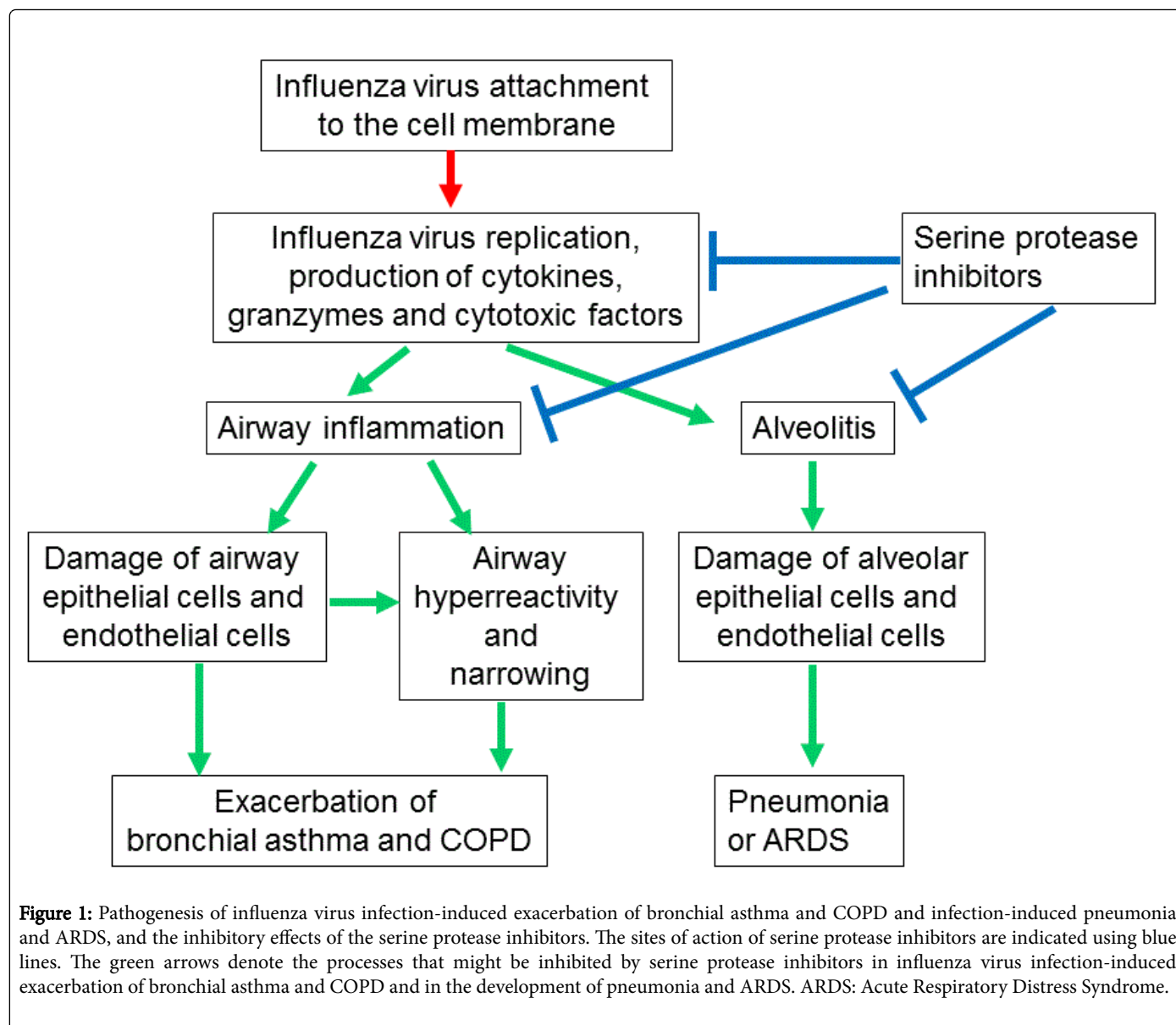


Figure 1: Pathogenesis of influenza virus infection-induced exacerbation of bronchial asthma and COPD and infection-induced pneumonia and ARDS, and the inhibitory effects of the serine protease inhibitors. The sites of action of serine protease inhibitors are indicated using blue lines. The green arrows denote the processes that might be inhibited by serine protease inhibitors in influenza virus infection-induced exacerbation of bronchial asthma and COPD and in the development of pneumonia and ARDS. ARDS: Acute Respiratory Distress Syndrome.

Furthermore, the expression of a trypsin-like protease was identified in rat bronchiolar Clara cells [64]. We identified the TMPRSS2 and TMPRSS11D protein at the cell membrane and in the cytoplasm of primary cultures of human tracheal epithelial cells using indirect immunofluorescence assay (Figure 2) [12]. We also detected mRNA encoding TMPRSS2, TMPRSS4 and TMPRSS11D in the cells [12]. Our findings pertaining to the expression of TMPRSS2, TMPRSS4 and TMPRSS11D in primary cultures of human tracheal epithelial cells [12] are consistent with the expression observed in previous reports.

Structure of TMPRSSs and their functions other than influenza virus activation

TMPRSS2, TMPRSS4 and TMPRSS11D belong to the type II transmembrane serine protease (TTSP) family, which was identified as a new family of S1 class serine proteases. TTSPs are characterized by an N-terminal transmembrane domain that anchors the enzymes to the plasma membrane, a stem region, and a catalytic domain of the

chymotrypsin S1 type. The most prominent member of the TTSP family is the digestive enzyme enteropeptidase, which was originally isolated as a soluble protease and was later shown to be the catalytic domain of the membrane-bound protease [65]. TTSPs are synthesized as single-chain zymogens that are activated into a mature form by C-terminal cleavage of a highly conserved arginine or lysine residue.

The physiological functions of TMPRSS2, TMPRSS4 and TMPRSS11D, other than proteolytic activation of influenza virus HA, have not been completely identified. However, TMPRSS2 also activates the spike protein of the severe acute respiratory syndrome coronavirus (SARS-CoV) on the cell surface [66], and camostat inhibits TMPRSS2-dependent infection by SARS-CoV [67]. The serine protease inhibitor aprotinin inhibits mucin production by an airway epithelial cell line through its action on TMPRSS11D [68]. Camostat and aprotinin inhibit sodium channel function in human bronchial epithelial cells, which can be reversed by saturating concentrations of trypsin [69], although the specific TMPRSSs affecting the channel function have not been identified. A variety of functions are attributed to TMPRSSs,

including prostate carcinogenesis and regulation of airway surface fluid volume by proteolytic cleavage of sodium channels by TMPRSS2 [57,70]; tissue development, cell differentiation, cancer progression

and metastasis by TMPRSS4 [60,71,72]; and prevention of fibrin deposition in the airway lumen by TMPRSS11D [73].

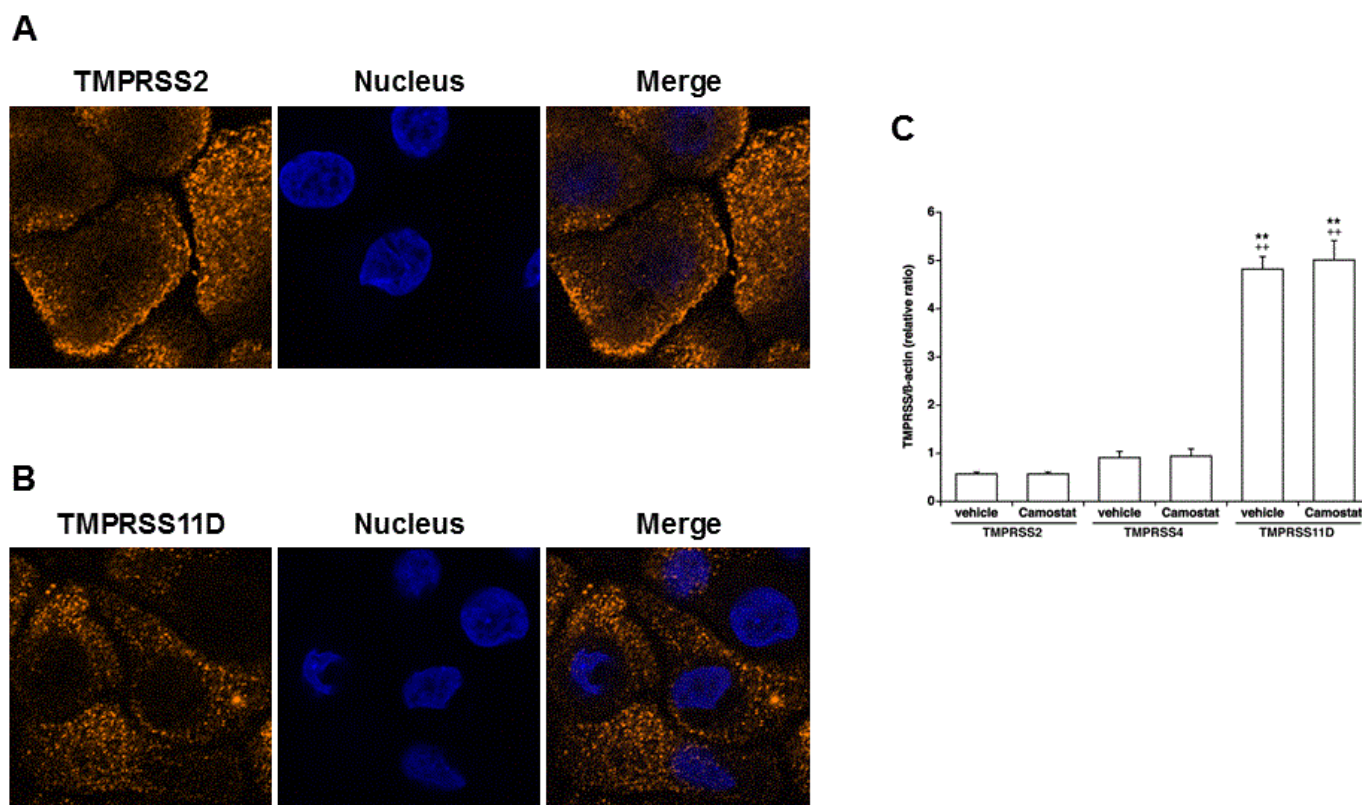


Figure 2: A and B: Indirect immunofluorescence staining of TMPRSS2 (A) and TMPRSS11D (B) in primary cultures of human tracheal epithelial (HTE) cells. TMPRSS2 and TMPRSS11D are stained orange at the cell membrane and in the cytoplasm. Nuclei are stained blue. Magnification: x 630. **C:** Expression of TMPRSS2 mRNA, TMPRSS4 mRNA and TMPRSS11D mRNA in HTE cells treated with camostat (10 mg/ml) or the vehicle control (1% water). The results are expressed as the ratio of TMPRSSs (TMPRSS2, TMPRSS4 or TMPRSS11D) mRNA expression compared with β -actin mRNA and are reported as the mean \pm SEM (n=3). Significant differences compared to the values of TMPRSS2 in the cells treated with vehicle alone (Vehicle) are indicated by **p<0.01. Significant differences compared to the values of TMPRSS4 in the cells treated with vehicle alone (Vehicle) are indicated by ++p<0.01. Camostat did not affect the expression of mRNA of three types of TMPRSSs [12].

Roles of serine proteases in influenza virus replication

The first report by Homma et al. [1] and subsequently published reports demonstrated that trypsin cleaved the surface glycoproteins of viruses (such as the fusion protein of Sendai virus and HA of the influenza virus), and the cleaved proteins caused fusion of the viral envelope to the host cell membrane, which is required for the spread of infection and pathogenicity [1-4].

Furthermore, the role of host serine proteases, including TMPRSS2, TMPRSS4 and TMPRSS11D, in the proteolytic activation of influenza virus HA and viral replication have been demonstrated using A549, 293T and Caco-2 cell lines transiently expressing the proteases TMPRSS2, TMPRSS4, and TMPRSS11D [6,58,74]. The cleavage site of influenza HA is located in a loop that protrudes prominently from the viral surface [75]. The subunit HA1 of A/Sendai-H/108/2009/(H1N1) pdm09 influenza virus, which is produced by cleaving the influenza virus precursor protein HA0, was detected in the supernatants of

primary cultures of human tracheal epithelial cells expressing TMPRSS2, TMPRSS4 and TMPRSS11D (Figure 3) [12]. TMPRSS2 and TMPRSS11D were detected at the cell membrane and in the cytoplasm of human tracheal epithelial cells (Figure 2) [12]. A serine protease inhibitor, camostat, reduced the amount of HA1 in the supernatants (Figure 3). These findings are consistent with reports that host proteases promote proteolytic activation of influenza virus HA in the trans-Golgi network and/or plasma membrane (Table 1) [76,77]. Newly synthesized influenza virus with cleaved HA may bud from the epithelial cell membrane.

Staphylococcus aureus secretes a protease that exerts a decisive influence on the outcome of influenza virus infection in mice through cleavage-mediated activation of the viral HA [78]. Extracellular proteolytic activation of HA by trypsin Clara isolated from rat lungs was also reported [79]. In contrast, Zhirnov et al. demonstrated that cleavage of HA in human adenoid epithelial cells occurs intracellularly

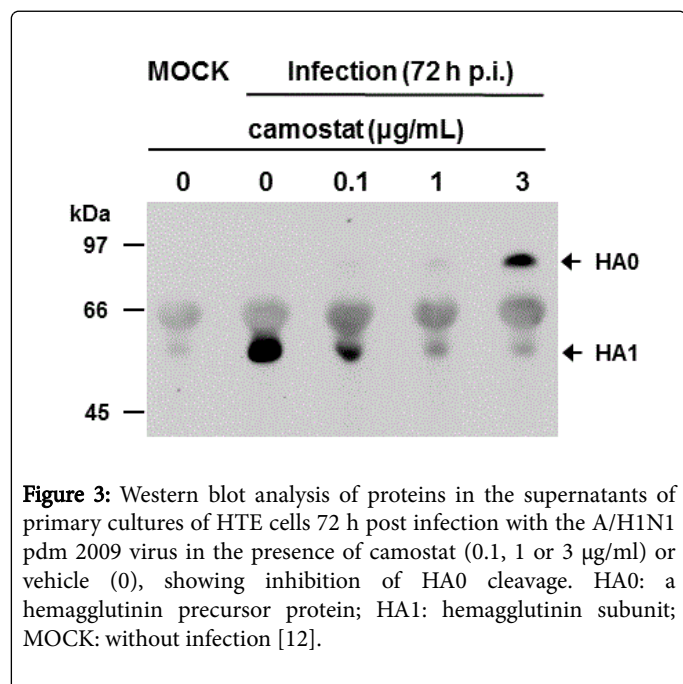
by cell-associated protease and that influenza virus is also activated intracellularly in human intestinal Caco-2 cells [10,77]. Furthermore, Böttcher-Friebertshäuser et al. [80] demonstrated that TMPRSS2 and TMPRSS11D cleaved newly synthesized HA before or during the budding of newly synthesized influenza virus. TMPRSS11D can also cleave the HA of incoming viruses prior to endocytosis at the surface of Madin Darby Canine Kidney (MDCK) cells transiently transfected

with these proteases (Table 1). In contrast, the authors reported low levels of enzymatic activity for soluble TMPRSS2 and TMPRSS11D in the supernatants of MDCK cells [80]. Thus, the site of influenza virus HA activation in human airway epithelial cells *in vivo* is still uncertain, and influenza virus HA activation may also occur outside of cells by soluble proteases released by host cells.

Localization			
TMPRSSs	Cell type or tissue localization of TMPRSS protein and mRNA	Reference	Year
TMPRSS2	Human lung (mRNA)	[56]	1999
	Human nasal, tracheal and distal airways (mRNA)	[57]	2002
	Human alveolar epithelial cells (mRNA)*	[6]	2006
	Caco-2 and Huh-7 cell line (protein and mRNA)*	[58]	2010
	Swine airway (protein and mRNA)	[59]	2014
	Human tracheal epithelial cells (protein and mRNA)	[12]	2015
TMPRSS4	Human lung cancer tissue (mRNA)	[60]	2008
	Caco-2 and Huh-7 cell line (mRNA)*	[58]	2010
	Mouse lung (mRNA)	[61]	2011
	Human tracheal epithelial cells (protein and mRNA)	[12]	2015
TMPRSS11D	Human bronchi and trachea (protein)	[62]	1997
	Human trachea (mRNA)	[63]	1998
	Swine airway (protein and mRNA)	[59]	2014
	Human tracheal epithelial cells (protein and mRNA)	[12]	2015
Proteolytic activation of influenza			
TMPRSSs	Cells	Reference	Year
TMPRSS2 and 11D	A549 cell line	[6]	2006
TMPRSS2 and 4	293T cell line	[74]	2009
TMPRSS2 and 4	Caco-2 cell line	[58]	2010
TMPRSS2, 4 and 11D	Human tracheal epithelial cells	[12]	2015
Site of proteolytic activation of influenza			
TMPRSSs	Site	Reference	Year
-	The late stages of intracellular transport in the trans-Golgi and plasma membrane	[77]	2003
TMPRSS2 (1) and 11D (1 and 2)	(1) Before or during budding; (2) Incoming viruses prior to endocytosis at the surface of cells	[80]	2010
TMPRSS2, 4 and 11D	Before or during budding	[12]	2015
Inhibitory effects of serine protease inhibitors on influenza virus replication			
Inhibitors	Cell type or tissue	Reference	Year
Aprotinin	Chicken embryo	[82]	1985
	MDCK cells	[8]	1992

	Human adenoid epithelial cells	[10]	2002
	Human tracheal epithelial cells	[12]	2015
Camostat	MDCK cells	[8]	1992
	Mouse airway, MDCK cells	[9]	1996
	Human tracheal epithelial cells	[12]	2015
Gabexate	MDCK cells	[8]	1992
	Human tracheal epithelial cells	[12]	2015
Leupeptin	Mouse lung	[83]	1987
	MDCK cells	[8]	1992
	Human adenoid epithelial cells	[10]	2002
Nafamostat	MDCK cells	[8]	1992
AEBSF and pAB	Mouse lung	[61]	2011

Table 1: TMPRSSs: Localization, activation, cleavage site, and inhibitors of influenza virus. AEBSF: 4-(2-aminoethyl)-benzolsulfonylfluoride; A549: human type II lung cell line; Caco-2: human colon cancer cell line; Huh-7: human hepatocellular carcinoma cell line; MDCK: Madin Darby Canine Kidney; pAB: p-aminobenzidine; TMPRSS: transmembrane protease serine S1 member. *Replication of influenza viruses and/or cleavage of influenza virus HA were examined in the cells co-transfected with plasmids encoding TMPRSSs.



Inhibitory effects of serine protease inhibitors on influenza virus replication

The serine protease inhibitor aprotinin and similar agents, such as leupeptin and camostat, suppress virus HA cleavage and reduce the replication of influenza viruses with a single arginine in the HA cleavage site [7]. Protease inhibitors, including ϵ -aminocaproic acid, can substantially reduce fatality in mice after influenza virus infection [81]. Similarly, viral replication in human adenoid epithelial cells was inhibited by aprotinin [10]. The protease inhibitors I-1, I-2 and I-3

inhibit influenza HA cleavage in MDCK cells co-transfected with plasmids encoding TMPRSS2 and TMPRSS11D [80]. Similarly, the protease inhibitors 4-(2-aminoethyl)-benzolsulfonylfluoride (AEBSF) and p-aminobenzidine (pAB) decrease influenza virus replication in mouse lung cells expressing proteases, including TMPRSS4 [61]. Furthermore, serine protease inhibitors, including camostat and aprotinin, inhibit HA cleavage and influenza virus replication in human tracheal epithelial cells that express TMPRSS2, 4 and 11D [12]. The inhibitory effects of protease inhibitors, such as aprotinin, camostat, gabexate, leupeptin and nafamostat, on influenza virus replication have also been reported in MDCK cells, human airway epithelial cells, chicken embryos and mice (Table 1) [7-10,12,82,83].

Camostat, a serine protease inhibitor used to treat patients with pancreatitis [84], reduced the replication of the influenza A/H1N1 pdm 2009 and A/H3N2 viruses in primary human tracheal epithelial cells as evaluated by viral titers and viral RNA levels (Table 2) [12]. Other types of serine protease inhibitors, such as aprotinin, gabexate and sivelestat, also reduced viral titers and/or RNA levels in the cells.

Thus, a variety of serine protease inhibitors reduce influenza virus replication in human and mouse airway and alveolar epithelial cells (Table 2). Serine protease inhibitors may prevent the exacerbation of bronchial asthma and COPD and the development of pneumonia and ARDS by reducing viral replication (Figure 1).

The effects of serine protease inhibitors on inflammatory cytokine release

IL-6 and TNF- α are associated with disease symptoms and severity in influenza-infected patients [39,85] and with cell damage [40]. Camostat and gabexate inhibit lipopolysaccharide-induced TNF- α release from macrophages [86] and the development of influenza-associated pneumonia in mice by inhibiting the production of cytokine, including IL-6 [87]. Aprotinin reduces ICAM-1 expression in human umbilical vein endothelial cells [88] and myocardial IL-6 gene

expression after cardiac ischemia and reperfusion in rats [89]. Serine protease inhibitors, including camostat, reduce production of IL-6 and TNF- α in human tracheal epithelial cells (Table 2) [12]. Furthermore, treatment of epithelial cells with an anti-IL-6 antibody attenuated cell damage in this model [40]. These findings suggest that the serine protease inhibitors may have anti-inflammatory effects in the lung, airways and other organs of patients with an influenza virus infection.

	Aprotinin	Camostat	Gabexate	Sivelestat
Viral release	++	+++	+	NS
Viral RNA replication	++	+++	+	+
IL-6	+	++	NS	NS
TNF- α	+	++	NS	NS

Table 2: Inhibitory effects of serine protease inhibitors on viral release, RNA replication, and cytokine release after influenza A/H1N1 pdm 2009 virus infection. A/H1N1 pdm 2009: Influenza A/H1N1 pdm 2009 virus; Viral release and RNA replication: The viral titers in the supernatants and viral RNA replication in the primary cultures of human tracheal epithelial cells 5 days after exposure to the influenza A/H1N1 pdm 2009 virus in the presence of camostat (10 mg/ml), sivelestat (10 mg/ml), gabexate (10 mg/ml), aprotinin (1000 KIU/ml) or the vehicle control; Cytokine release: The concentration of IL-6 and TNF- α in the supernatants before or 5 days after exposure to the influenza A/H1N1 pdm 2009 virus in the presence of camostat (10 mg/ml), sivelestat (10 mg/ml), gabexate (10 mg/ml), aprotinin (1000 KIU/ml) or the vehicle control. Significant differences from the viral infection alone are indicated by + p <0.05, ++ p <0.01 and +++ p <0.001. NS: Not significant [12].

In addition to the reduction of viral replication, serine protease inhibitors may provide clinical benefits by preventing the exacerbation of bronchial asthma and COPD and the development of pneumonia and ARDS, through reducing production of cytokines, granzymes and other types of cytotoxic factors (Figure 1).

Both neuraminidase inhibitors and serine protease inhibitors reduce the production of inflammatory cytokines *in vitro* [12,27]. Therefore, the combined use of the two inhibitors may enhance the inhibitory effects on cytokine production and may serve as a more efficient anti-viral therapy. However, because physicians usually prescribed only one type of neuraminidase inhibitor for treatment, these serine protease inhibitors may be used alone at first, even in cases when these inhibitors can be clinically used. The effects of combination therapies involving serine protease inhibitors and other types of drugs, such as neuraminidase inhibitors and a polymerase inhibitor, should be elucidated.

The effects of granzymes and their inhibitors on inflammation and influenza virus infection

Because granzymes are granule-stored lymphocyte serine proteases and are produced in CD8⁺ cells in response to influenza virus replication [41], it could be speculated that serine protease inhibitors inhibit the cytotoxic activities of granzymes themselves and reduce CD8⁺ cell activation and granzyme production in response to virus replication. Serine protease inhibitors reduce the production of cytokines, including IL-6 [12], that induce granzyme production [42]. Thus, serine protease inhibitors could inhibit the cytotoxic activities of

granzymes produced by immune cells during influenza virus replication through these mechanisms. However, the effects of granzymes and other serine proteases and their inhibitors on inflammation in response to influenza virus infection are unclear.

Aprotinin has been shown to inhibit the L-glutamyl-2-naphthylamidase activity of granzyme B in a human cytolytic lymphocyte line, Q31, whereas leupeptin does not have a significant inhibitory effect [90]. In contrast, Zhong et al. reported that granzyme K inhibited replication of influenza virus by cleaving the nuclear transport complex (the importin α 1/ β dimer) of infected host cells [91]. However, the effects of granzymes and other serine proteases and their inhibitors on virus entry, including the proteolytic activities of influenza HA, are still unclear. Further studies are required to clarify these effects.

Potency of serine protease inhibitors

Of all the protease inhibitors studied (aprotinin, camostat, gabexate and sivelestat) in human tracheal epithelial cells, camostat was the most potent inhibitor of influenza virus replication and inflammatory cytokine production (Table 2) [12]. This is supported by the similar levels of potency observed in a report by Hosoya et al. using MDCK cells [8]. Observations of the MDCK influenza infection model suggest that nafamostat may be more potent than camostat and that leupeptin and pepstatin are less effective than aprotinin [8]. Thus, of the protease inhibitors studied, camostat and nafamostat may have the highest potential for reducing influenza virus replication and/or inflammatory cytokine production (Table 2) [8,12]. However, the exact difference in the potency between camostat and nafamostat has not been examined in human airway epithelial cells.

Conclusion

The serine proteases of host cells induce the proteolytic activation of influenza virus HA, and serine protease inhibitors reduce the replication of the influenza virus within, and the production of inflammatory cytokines by, human airway epithelial cells. *In vitro* models suggest that serine protease inhibitors are a potential treatment for influenza patients, but animal experiments are required to confirm their anti-viral effects and low toxicity. Ultimately, clinical trials in influenza-infected patients will be needed to assess the clinical benefits of these inhibitors.

Acknowledgement

This study was supported by a Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 24659398 and 25293189 and Ono Yakuhin Co., Ltd.

Conflicts of Interest

All authors have no conflict of interest. Yamaya is a professor in the Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine. This department had been funded by eleven pharmaceutical companies until March 31, 2014, including Ono Yakuhin Co., Ltd. which provided camostat mesilate, sivelestat and gabexate mesilate. From April 1, 2014, this department is funded by eight pharmaceutical companies, which are as follows: Kyorin Pharmaceutical Co. Ltd., Abott Japan, Co., Ltd., Taisho Toyama Pharmaceutical Co., Ltd., AstraZeneca Co. Ltd, Otsuka

Pharmaceutical Co. Ltd., Teijin Pharma Co., Ltd., Toyama Chemical Co., Ltd., and Nippon Boehringer-Ingelheim Co., Ltd.

References

1. Homma M (1971) Trypsin action on the growth of Sendai virus in tissue culture cells. I. Restoration of the infectivity for L cells by direct action of trypsin on L cell-borne Sendai virus. *J Virol* 8: 619-629.
2. Ohuchi M, Homma M (1976) Trypsin action on the growth of Sendai virus in tissue culture cells. IV. Evidence for activation of sendai virus by cleavage of a glycoprotein. *J Virol* 18: 1147-1150.
3. Nagai Y (1993) Protease-dependent virus tropism and pathogenicity. *Trends Microbiol* 1: 81-87.
4. Klenk HD, Garten W (1994) Host cell proteases controlling virus pathogenicity. *Trends Microbiol* 2: 39-43.
5. Klenk HD, Rott R, Orlich M, Blödorn J (1975) Activation of influenza A viruses by trypsin treatment. *Virology* 68: 426-439.
6. Böttcher E, Matrosovich T, Beyerle M, Klenk HD, Garten W, et al. (2006) Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. *J Virol* 80: 9896-9898.
7. Zhirnov OP, Klenk HD, Wright PF (2011) Aprotinin and similar protease inhibitors as drugs against influenza. *Antiviral Res* 92: 27-36.
8. Hosoya M, Matsuyama S, Baba M, Suzuki H, Shigeta S (1992) Effects of protease inhibitors on replication of various myxoviruses. *Antimicrob Agents Chemother* 36: 1432-1436.
9. Lee MG, Kim KH, Park KY, Kim JS (1996) Evaluation of anti-influenza effects of camostat in mice infected with non-adapted human influenza viruses. *Arch Virol* 141: 1979-1989.
10. Zhirnov OP, Ikizler MR, Wright PF (2002) Cleavage of influenza A virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. *J Virol* 76: 8682-8689.
11. Yamaya M, Finkbeiner WE, Chun SY, Widdicombe JH (1992) Differentiated structure and function of cultures from human tracheal epithelium. *Am J Physiol* 262: L713-724.
12. Yamaya M, Shimotai Y, Hatachi Y, Lusamba Kalonji N, Tando Y, et al. (2015) The serine protease inhibitor camostat inhibits influenza virus replication and cytokine production in primary cultures of human tracheal epithelial cells. *Pulm Pharmacol Ther* 33: 66-74.
13. Murray CJ, Lopez AD (1996) Evidence-based health policy--lessons from the Global Burden of Disease Study. *Science* 274: 740-743.
14. Fukuchi Y, Nishimura M, Ichinose M, Adachi M, Nagai A, et al. (2004) COPD in Japan: the Nippon COPD Epidemiology study. *Respirology* 9: 458-465.
15. Buist AS, Vollmer WM (1994) Smoking and other risk factors. In: Murray JF, Nadel JA (eds.) *Textbook of respiratory Medicine*. WB Sanders, Philadelphia, USA.
16. Hayden FG, Gwaltney JM. Jr (1988) Viral infections: *Textbook of Respiratory Medicine*. In: Murray J, Nadel JA (eds.) WB Saunders Company, Philadelphia, USA.
17. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, et al. (1995) Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 310: 1225-1229.
18. Nichol KL, Margolis KL, Wuorenma J, Von Sternberg T (1994) The efficacy and cost effectiveness of vaccination against influenza among elderly persons living in the community. *N Engl J Med* 331: 778-784.
19. Negri E, Colombo C, Giordano L, Groth N, Apolone G, et al. (2005) Influenza vaccine in healthy children: a meta-analysis. *Vaccine* 23: 2851-2861.
20. DiazGranados CA, Denis M, Plotkin S (2012) Seasonal influenza vaccine efficacy and its determinants in children and non-elderly adults: a systemic review with meta-analyses of controlled trials. *Vaccine* 31: 49-57.
21. Beigel J, Bray M (2008) Current and future antiviral therapy of severe seasonal and avian influenza. *Antiviral Res* 78: 91-102.
22. Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis RR, et al. (2000) Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. *US Oral Neuraminidase Study Group. JAMA* 283: 1016-1024.
23. Kumar A (2011) Early versus late oseltamivir treatment in severely ill patients with 2009 pandemic influenza A (H1N1): speed is life. *J Antimicrob Chemother* 66: 959-963.
24. Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, Hernandez M, Quiñones-Falconi F, et al. (2009) Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. *N Engl J Med* 361: 680-689.
25. Saitoh M, Wajima N, Yamaya M (2015) An accumulation of air and a retroperitoneal abscess during type B influenza virus infection in an elderly adult. *Geriatr Gerontol Int* 15: 668-669.
26. Gooskens J, Jonges M, Claas EC, Meijer A, van den Broek PJ, et al. (2009) Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A (H1N1) virus. *JAMA* 301: 1042-1046.
27. Yamaya M, Kalonji N, Kubo H, Saito K, Saito R, et al. (2015) The effects of neuraminidase inhibitors on the release of oseltamivir-sensitive and oseltamivir-resistant influenza viruses from primary cultures of human tracheal epithelium. *J Med Virol* 87: 25-34.
28. Shobugawa Y, Saito R, Sato I, Kawashima T, Dapat C, et al. (2012) Clinical effectiveness of neuraminidase inhibitors - oseltamivir, zanamivir, laninamivir, and peramivir - for treatment of influenza A (H3N2) and A (HiN1)pdm09 infection: an observational study in the 2010-2011 influenza season in Japan. *J Infect Chemother* 18: 858-864.
29. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, et al. (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* 360: 1831-1837.
30. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, et al. (2006) Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 12: 1203-1207.
31. Lipatov AS, Andreansky S, Webby RJ, Hulse DJ, Rehgl JE, et al. (2005) Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. *J Gen Virol* 86: 1121-1130.
32. Mauad T, Hajjar LA, Callegari GD, da Silva LF, Schout D, et al. (2010) Lung pathology in fatal novel human influenza A (H1N1) infection. *Am J Respir Crit Care Med* 181: 72-79.
33. Ruwanpura SM, McLeod L, Miller A, Jones J, Bozinovski S, et al. (2011) Interleukin-6 promotes pulmonary emphysema associated with apoptosis in mice. *Am J Respir Cell Mol Biol* 45: 720-730.
34. Wang S, Le TQ, Kurihara N, Chida J, Cisse Y, et al. (2010) Influenza virus-cytokine-protease cycle in the pathogenesis of vascular hyperpermeability in severe influenza. *J Infect Dis* 202: 991-1001.
35. Nicholson KG, Kent J, Ireland DC (1993) Respiratory viruses and exacerbations of asthma in adults. *BMJ* 307: 982-986.
36. Rohde G, Wiethage A, Borg I, Kauth M, Bauer TT, et al. (2003) Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax* 58: 37-42.
37. Yasuda H, Suzuki T, Zayasu K, Ishizuka S, Kubo H, et al. (2005) Inflammatory and bronchospastic factors in asthma exacerbations caused by upper respiratory tract infections. *Tohoku J Exp Med* 207: 109-118.
38. Gao R, Bhatnagar J, Blau DM, Greer P, Rollin DC, et al. (2013) Cytokine and chemokine profiles in lung tissues from fatal cases of 2009 pandemic influenza A (H1N1): role of the host immune response in pathogenesis. *Am J Pathol* 183: 1258-1268.
39. Paquette SG, Banner D, Zhao Z, Fang Y, Huang SS, et al. (2012) Interleukin-6 is a potential biomarker for severe pandemic H1N1 influenza A infection. *PLoS One* 7: e38214.
40. Yamaya M, Nadine LK, Ota C, Kubo H, Makiguchi T, et al. (2014) Magnitude of influenza virus replication and cell damage is associated with interleukin-6 production in primary cultures of human tracheal epithelium. *Respir Physiol Neurobiol* 202: 16-23.
41. Piet B, de Bree GJ, Smids-Dierdorff BS, van der Loos CM, Remmerswaal EB, et al. (2011) CD8+ T cells with an intraepithelial phenotype

- upregulate cytotoxic function upon influenza infection in human lung. *J Clin Invest* 121: 2254-2263.
42. Böttcher JP, Schanz O, Garbers C, Zaremba A, Hegenbarth S, et al. (2014) IL-6 trans-signaling-dependent rapid development of cytotoxic CD8+ T cell function. *Cell Rep* 8: 1318-1327.
 43. US Department of Health and Human Services (2015) Global Initiative for Asthma: Global Strategy for Asthma Management and Prevention 2015. National Institutes of Health, National Heart, Lung, and Blood Institute.
 44. Celli BR, MacNee W; ATS/ERS Task Force (2004) Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *Eur Respir J* 23: 932-946.
 45. Davies L, Angus RM, Calverley PM (1999) Oral corticosteroids in patients admitted to hospital with exacerbations of chronic obstructive pulmonary disease: a prospective randomized controlled trial. *Lancet* 354: 456-460.
 46. Ram FS, Rodriguez-Roisin R, Granados-Navarrete A, Garcia-Aymerich J, Barnes NC (2011) Antibiotics for exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*.
 47. Rogers GN, Paulson JC (1983) Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 127: 361-373.
 48. Couceiro JN, Paulson JC, Baum LG (1993) Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium: the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res* 29: 155-165.
 49. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, et al. (2006) Avian flu: influenza virus receptors in the human airway. *Nature* 440: 435-436.
 50. Yamaya M, Shinya K, Hatachi Y, Kubo H, Asada M, et al. (2010) Clarithromycin inhibits type A seasonal influenza virus infection in human airway epithelial cells. *J Pharmacol Exp Ther* 333: 81-90.
 51. Yamaya M, Nishimura H, Shinya K, Hatachi Y, Sasaki T, et al. (2010) Inhibitory effects of carbocisteine on type A seasonal influenza virus infection in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 299: L160-L168.
 52. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 101: 4620-4624.
 53. Palese P, Shaw ML (2006) Orthomyxoviridae: Fields' Virology. In: Knipe DM, and Howley PM (eds.) (5th edn.) Lippincott Williams and Wilkins, Philadelphia, USA.
 54. Siczekarski SB, Brown HA, Whittaker GR (2003) Role of protein kinase C betaII in influenza virus entry via late endosomes. *J Virol* 77: 460-469.
 55. White J, Kielian M, Helenius A (1983) Membrane fusion proteins of enveloped animal viruses. *Q Rev Biophys* 16: 151-195.
 56. Lin B, Ferguson C, White JT, Wang S, Vessella R, et al. (1999) Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. *Cancer Res* 59: 4180-4184.
 57. Donaldson SH, Hirsh A, Li DC, Holloway G, Chao J, et al. (2002) Regulation of the epithelial sodium channel by serine proteases in human airways. *J Biol Chem* 277: 8338-8345.
 58. Bertram S, Glowacka I, Blazejewska P, Soilleux E, Allen P, et al. (2010) TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. *J Virol* 84: 10016-10025.
 59. Peitsch C, Klenk HD, Garten W, Böttcher-Friebertshäuser E (2014) Activation of influenza A viruses by host proteases from swine airway epithelium. *J Virol* 88: 282-291.
 60. Jung H, Lee KP, Park SJ, Park JH, Jang YS, et al. (2008) TMPRSS4 promotes invasion, migration and metastasis of human tumor cells by facilitating an epithelial-mesenchymal transition. *Oncogene* 27: 2635-2647.
 61. Bahgat MM, Blazejewska P, Schughart K (2011) Inhibition of lung serine proteases in mice: a potentially new approach to control influenza infection. *Viol J* 8: 27.
 62. Yasuoka S, Ohnishi T, Kawano S, Tsuchihashi S, Ogawara M, et al. (1997) Purification, characterization, and localization of a novel trypsin-like protease found in the human airway. *Am J Respir Cell Mol Biol* 16: 300-308.
 63. Yamaoka K, Masuda K, Ogawa H, Takagi K, Umemoto N, et al. (1998) Cloning and characterization of the cDNA for human airway trypsin-like protease. *J Biol Chem* 273: 11895-11901.
 64. Kido H, Yokogoshi Y, Sakai K, Tashiro M, Kishino Y, et al. (1992) Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. *J Biol Chem* 267: 13573-13579.
 65. Szabo R, Bugge TH (2008) Type II transmembrane serine proteases in development and disease. *Int J Biochem Cell Biol* 40: 1297-1316.
 66. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, et al. (2010) Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol* 84: 12658-12664.
 67. Kawase M, Shirato K, van der Hoek L, Taguchi F, Matsuyama S (2012) Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. *J Virol* 86: 6537-6545.
 68. Chokki M, Yamamura S, Eguchi H, Masegi T, Horiuchi H, et al. (2004) Human airway trypsin-like protease increases mucin gene expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 30: 470-478.
 69. Coote K, Atherton-Watson HC, Sugar R, Young A, MacKenzie-Beevor A, et al. (2009) Camostat attenuates airway epithelial sodium channel function in vivo through the inhibition of a channel-activating protease. *J Pharmacol Exp Ther* 329: 764-774.
 70. Tomlins SA, Bjartell A, Chinnaiyan AM, Jenster G, Nam RK, et al. (2009) ETS gene fusions in prostate cancer: from discovery to daily clinical practice. *Eur Urol* 56: 275-286.
 71. Ohler A, Becker-Pauly C (2011) Morpholino knockdown of the ubiquitously expressed transmembrane serine protease TMPRSS4s in zebrafish embryos exhibits severe defects in organogenesis and cell adhesion. *Biol Chem* 392: 653-664.
 72. Ohler A, Becker-Pauly C (2012) TMPRSS4 is a type II transmembrane serine protease involved in cancer and viral infections. *Biol Chem* 393: 907-914.
 73. Yoshinaga S, Nakahori Y, Yasuoka S (1998) Fibrinogenolytic activity of a novel trypsin-like enzyme found in human airway. *J Med Invest* 45: 77-86.
 74. Chaipan C, Kobasa D, Bertram S, Glowacka I, Steffen I, et al. (2009) Proteolytic activation of the 1918 influenza virus hemagglutinin. *J Virol* 83: 3200-3211.
 75. Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, et al. (1998) Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* 95: 409-417.
 76. Böttcher-Friebertshäuser E, Klenk HD, Garten W (2013) Activation of influenza viruses by proteases from host cells and bacteria in the human airway epithelium. *Pathog Dis* 69: 87-100.
 77. Zhirnov OP, Vorobjeva IV, Ovcharenko AV, Klenk HD (2003) Intracellular cleavage of human influenza A virus hemagglutinin and its inhibition. *Biochemistry (Mosc)* 68: 1020-1026.
 78. Tashiro M, Ciborowski P, Klenk HD, Pulverer G, Rott R (1987) Role of Staphylococcus protease in the development of influenza pneumonia. *Nature* 325: 536-537.
 79. Kido H, Murakami M, Oba K, Chen Y, Towatari T (1999) Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. *Mol Cells* 9: 235-244.
 80. Böttcher-Friebertshäuser E, Freuer C, Sielaff F, Schmidt S, Eickmann M, et al. (2010) Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. *J Virol* 84: 5605-5614.

81. Zhirnov OP, Ovcharenko AV, Bukrinskaya AG (1982) Protective effect of protease inhibitors in influenza virus infected animals. *Arch Virol* 73: 263-272.
82. Zhirnov OP, Ovcharenko AV, Bukrinskaya AG (1985) Myxovirus replication in chicken embryos can be suppressed by aprotinin due to the blockage of viral glycoprotein cleavage. *J Gen Virol* 66 : 1633-1638.
83. Tashiro M, Klenk HD, Rott R (1987) Inhibitory effect of a protease inhibitor, leupeptin, on the development of influenza pneumonia, mediated by concomitant bacteria. *J Gen Virol* 68 : 2039-2041.
84. Hiraku S, Muryobayashi K, Ito H, Inagawa T, Tuboshima M (1982) Absorption and excretion of camostat (FOY-305) orally administered to male rabbit and healthy subjects. *Iyaku Kenkyu* 13: 756-765.
85. Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, et al. (1998) Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. *J Clin Invest* 101: 643-649.
86. Tumurkhuu G, Koide N, Hiwasa T, Ookoshi M, Dagvadorj J, et al. (2011) ONO 340, a synthetic serine protease inhibitor, inhibits lipopolysaccharide-induced tumor necrosis factor- α and nitric oxide production and protects mice from lethal endotoxic shock. *Innate Immun* 17: 97-105.
87. Kosai K, Seki M, Yanagihara K, Nakamura S, Kurihara S, et al. (2008) Gabexate mesilate suppresses influenza pneumonia in mice through inhibition of cytokines. *J Int Med Res* 36: 322-328.
88. Asimakopoulos G, Lidington EA, Mason J, Haskard DO, Taylor KM, et al. (2001) Effect of aprotinin on endothelial cell activation. *J Thorac Cardiovasc Surg* 122: 123-128.
89. Buerke M, Pruefer D, Sankat D, Carter JM, Buerke U, et al. (2007) Effects of aprotinin on gene expression and protein synthesis after ischemia and reperfusion in rats. *Circulation* 116: 1121-1126.
90. Poe M, Blake JT, Boulton DA, Gammon M, Sigal NH, et al. (1991) Human cytotoxic lymphocyte granzyme B. Its purification from granules and the characterization of substrate and inhibitor specificity. *J Biol Chem* 266: 98-103.
91. Zhong C, Li C, Wang X, Toyoda T, Gao G, et al. (2012) Granzyme K inhibits replication of influenza virus through cleaving the nuclear transport complex importin α 1/ β dimer of infected host cells. *Cell Death Differ* 19: 882-890.