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Aprotinin and similar protease inhibitors as drugs against influenza ☆

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ABSTRACT

Efforts to develop new antiviral chemotherapeutic approaches are focusing on compounds that target either influenza virus replication itself or host factor(s) that are critical to influenza replication. Host protease mediated influenza hemagglutinin (HA) cleavage is critical for activation of virus infectivity and as such is a chemotherapeutic target. Influenza pathogenesis involves a "vicious cycle" in which host proteases activate progeny virus which in turn amplifies replication and stimulates further protease activities which may be detrimental to the infected host. Aprotinin, a 58 amino acid polypeptide purified from bovine lung that is one of a family of host-targeted antivirals that inhibit serine proteases responsible for influenza virus activation. This drug and similar agents, such as leupeptin and camostat, suppress virus HA cleavage and limit reproduction of human and avian influenza viruses with a single arginine in the HA cleavage site. Site-directed structural modifications of aprotinin are possible to increase its intracellular targeting of cleavage of highly virulent H5 and H7 hemagglutinins possessing multi-arginine/lysine cleavage site. An additional mechanism of action for serine protease inhibitors is to target a number of host mediators of inflammation and down regulate their levels in virus-infected hosts. Aprotinin is a generic drug approved for intravenous use in humans to treat pancreatitis and limit post-operative bleeding. As an antiinfluenzal compound, aprotinin might be delivered by two routes: (i) a small-particle aerosol has been approved in Russia for local respiratory application in mild-to-moderate influenza and (ii) a proposed intravenous administration for severe influenza to provide both an antiviral effect and a decrease in systemic pathology and inflammation.

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1. Introduction

Emergence of the novel "swine"-origin influenza H1N1 (H1N1pdm) pandemic stresses the need for the development of new antiviral drugs. Only two classes of specific antiviral compounds are currently in use in medical practice against influenza. These are amantadine derivatives (Symmetrel and Flumadin), which specifically block the influenza A virus ionic channel M2 (Pinto and Lamb, 2006), and oseltamivir (Tamiflu) and zanamivir (Relenza), which specifically suppress influenza A and B virus neuraminidases (Moscona, 2005). Unfortunately, emergence and spread of virus variants resistant to both M2- and NA-inhibitor drugs have been documented in influenza H1N1, H3N2, H1N1pdm, and H5N1 virus-infected patients (CDC, 2011; Moscona, 2009; Thorlund et al., 2011). The emergence of resistance without a major loss of virus fitness contributes to the need for alternative

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antivirals to combat modern epidemics of influenza. Research efforts are focusing on development of new antiviral chemotherapeutic approaches that target influenza virus replication itself or host factor(s) which are integral to virus replication, or interruption of virus-mediated pathogenesis in the host.

These chemotherapeutic principles are referred to as virus- and host-targeted antivirals. Virus-targeted agents inhibiting protein HA (Russell et al., 2008), NS1 (Walkiewicz et al., 2011), NP (Kao et al., 2010), polymerase proteins (Furuta et al., 2005; Ghanem et al., 2007), viral RNA survival (Wu et al., 2008) are in development. Host-targeted virus inhibitors include the NF-κB and Raf/MEK/ERK pathways (Ludwig, 2009), the ubiquitination process (Liao et al., 2010), expression of cellular sialic acid receptors (Belser et al., 2007; Malakhov et al., 2006; Zhang, 2008) and protease inhibitors – the subject of this review. A theoretical advantage of a host targeted antivirals, including protease inhibitors, is that resistance will not be selected for as readily as it is by influenza virus for anti-viral targets.

Of the protease inhibitors, there has been the greatest experience with aprotinin. Aprotinin is a serine protease inhibitor that effectively targets host trypsin-like protease(s) responsible for influenza virus hemagglutinin cleavage and virus activation in infected organism (Zhirnov, 1983). This anti-protease compound has a long history in medical practice in the treatment of pancreatitis and post-operative bleeding (Fritz and Wunderer, 1983; Trautschold et al., 1967). There are several licensed compounds of aprotinin, including Trasylol™ (Bayer AG, Germany), Antagosan™ (Sanofi Aventis, France), Gordox™ (Gedeon Richter, Hungary), and Contrycal™ (AWD, Germany). There is an influenza specific approval for topical use of aprotinin in Russia. Evaluation of aprotinin as a representative of the protease-inhibitor class of host-targeted anti-influenzal agents will be highlighted in this minireview.

2. Influenza virus proteolytic activation and host proteases involved in this process

More than 30 years ago it was shown that influenza viruses require activation by host specific protease(s) (influenza activating protease – IAP) to establish productive multicycle replication in tissue culture (Klenk et al., 1975; Lazarowitz and Choppin, 1975). This activation process is mediated by cleavage at a specific site of the virus hemagglutinin protein (HA). HA assembles as a homotrimer forming external spikes on the surface of the virion (Wilson et al., 1981). HA is synthesized as a precursor HA0 (~75 kD) and is proteolytically cleaved into two disulfide-linked fragments, HA1 (55 kD) and HA2 (20 kD) (see Fig. 1A). This point cleavage occurs at site in the HA molecule containing either a single arginine (Arg) (or rarely a lysine (Lys)) referred to as a mono-basic site or an Lys/Arg-X-Arg/Lys-Arg motif – a multi-basic site. A mono-basic site (Q/E-X-R where X is any amino acid except Cys or basic amino acids) is characteristic of the HA of all human H1–H3 subtypes, including H1N1pdm, and low pathogenic avian viruses of subtypes H1–H16. To date only highly virulent avian viruses of H5 and H7 but critically including "avian-origin" human H5N1 virus have been found to possess multi-basic cleavage sites (Garten and Klenk, 2009). The multibasic cleavage is associated with the capacity to be more promiscuously cleaved by a broader range of proteases with the result that systemic spread of influenza is possible and a resultant intense cytokine response appears to contribute to the severity of illness.

Crystallography has shown that the HA cleavage site is located in a loop formed by 19 amino acids of which 8 ones protrude externally from the surface of the HA trimer (Chen et al., 1998). Thus, the external presentation of the HA cleavable peptide contributes to its easy access for proteases. This protease accessibility is additionally controlled by two factors. First, an elongation of the loop by insertion of several amino acids upstream of the cleavage site is known to enhance protease accessibility and proteolytic cleavability of the HA (Khatchikian et al., 1989; Ohuchi et al., 1991). Second, carbohydrate chains located in the vicinity to the cleavage peptide in the HA molecule can significantly mask its recognition by target protease(s) (Kawaoka and Webster, 1989; Ohuchi et al., 1989). Due to these factors, protease accessibility and extent of the HA cleavage can vary from virus strain to strain.

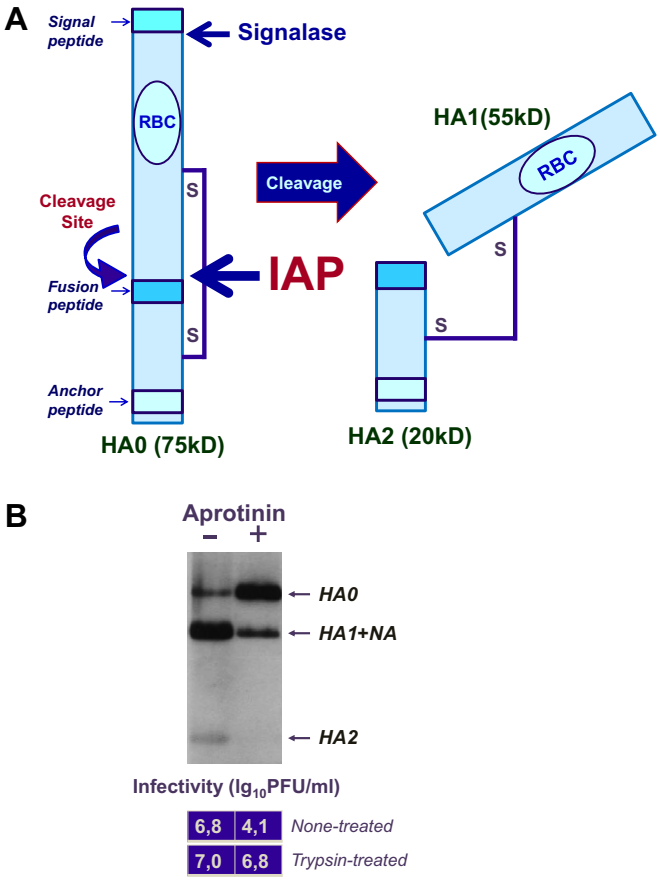

Fig. 1. Proteolytic cleavage of influenza A virus HA and its inhibition by aprotinin. (A) HA molecule is synthesized and glycosylated as precursor HA0 (mol. wt. 75 kD) interacting with endoplasmic reticulum through N-terminal signal and C-terminal anchor peptides. Signal peptide is removed by host Signalase and additionally HA0 is cleaved intracellularly by host trypsin-like (in monobasic site) or furin-like (in multibasic site) serine proteases into two subunits, HA1 (55 kD) and HA2 (20 kD). After the cleavage, the virus fusion peptide locates in the N-terminus of the HA2 and a receptor binding center (RBC), which is responsible for specific recognition of sialic acid-containing receptors on the surface of target cells, is formed by the HA1. (B) Human differentiated bronchial epithelium (HTBE cells) was infected with A/Hamburg/05/2009 (H1N1pdm) virus (MOI 1) and incubated without and with aprotinin (250 KIU per ml of culture fluid). 40 h post infection progeny virus accumulated in culture fluid was purified by differential centrifugation and studied in SDS-containing polyacrylamide gel electrophoresis. To discriminate between infectious (activated) and non-infectious (non-activated) forms of virus, culture fluid samples were assayed for infectivity by plaque focus formation in MDCK cells with and without trypsin pretreatment, as described (Zhirnov et al., 2011). Trypsin pretreatment of analyzed samples allowed evaluating the level of potential virus infectivity.

Cleavage of the HA is necessary to mediate fusion of viral and host cell membranes and to complete entry of the viral nucleic acid into the host cell cytoplasm. A hydrophobic "fusion" peptide released after cleavage at the N-terminus of the HA2 subunit plays a central role in this multistep entry process (see Fig. 1A). Initially, HA molecules attach virus to sialic acid containing receptors on cell surface and then virus is internalized in endosome by receptor

mediated endocytosis. After acidification in the cellular endosome and disassembly of virion ribonucleoprotein (RNP) from a protein matrix M1 (Zhirnov, 1990), the fusion HA peptide is released from the stem cavity in the cleaved HA1/HA2 polypeptide backbone and fuses endosomal and virion lipid membranes to form a membrane pore and open the entry of viral RNP into cytoplasm of infected cell (Pinto and Lamb, 2006). Thus, this intracellular delivery of viral genome is mediated at its final stage only by the cleaved HA1/HA2 protein to initiate productive infection. Effectively the point cleavage of HA0 increases infectivity of influenza virus by 1000 times and more (Zhirnov et al., 1982c). The proteolytic activation of influenza virus infectivity via the cleavage of HA0 into HA1/HA2 occurs in human tracheo-bronchial respiratory epithelium (HTBE) (Zhirnov et al., 2002, 2011). Its sensitivity to inhibitory action of aprotinin is illustrated in Fig. 1B. It is shown, that influenza virus synthesized in HTBE cells contained predominantly cleaved HA1/HA2 and characterized by high infectious activity, while in the presence of aprotinin, virus contained predominantly uncleaved HA0 and possessed about 500 times lower infectivity (Zhirnov et al., 2011). Exposure to trypsin significantly increases infectivity of aprotinin-treated virus thus confirming the non-activated HA0 in virus synthesized under aprotinin treatment.

Influenza virus HA cleavage events of mono-basic and multi-basic protease configurations are accomplished in different compartments in infected cells. HA0 of highly virulent Influenza viruses H5 and H7 are cleaved intracellularly in trans-Golgi membranes (“cleavage from within”) by ubiquitous proteases belonging to a family of eukaryotic calcium-dependent subtilisin-like serine endoproteases, such as furin, PC5/6 (Garten and Klenk, 2009). Until recently the trans-Golgi pathway was considered as a major compartment for multibasic HA cleavage. Alternative cleavage of multibasic HA0 can take place in cell plasma membrane. Recently identified type II transmembrane serine proteases, such as MSPL, and TMPRSS13, are suggested to accumulate in this cell compartment and specifically cleave the multibasic HA0 of highly virulent viruses (Okumura et al., 2010). Thus, all virions of the highly virulent viruses leave infected cells already in an activated form.

Accumulating data show that cleavage of the monobasic HA in differentiated epithelial cells including respiratory epithelium is associated with infected cells and occurs at the late stage in different compartments of exocytic way in a plasma membrane area and also qualifies as “cleavage from within” (Zhirnov et al., 2002; Zhirnov and Klenk, 2003). Thus, such cleavage is felt to be accomplished by cell-associated proteases in an endocrine fashion (Bertram et al., 2010; Böttcher-Friebertshäuser et al., 2010; Zhirnov et al., 2002; Zhirnov and Klenk, 2003). Importantly, this type of cleavage is less efficient with only 40–90% of virions released from the cell in an active form (Zhirnov et al., 2002, 2011; Zhirnov and Klenk, 2003). The fact, that influenza activating proteases candidates, such as epitheliasin (TMPRSS2), TMPRSS4, HAT, etc., are transmembrane proteases recirculating at the plasma membrane area (Hooper et al., 2001; Netzel-Arnett et al., 2003), is consistent with this concept.

The extracellular activation of monobasic influenza viruses by cellular secreted proteases, such as tryptase Clara, miniplasmin, ectopic anionic trypsin, mast cell tryptase (Kido et al., 1992; Murakami et al., 2001; Sato et al., 2003; Towatari et al., 2002), acting in a paracrine fashion remains an alternative or complementary mechanism for certain virus strains (Kido et al., 1996). However, based on respiratory epithelium or epithelium-like systems such extracellular activation seems to be less important (Böttcher-Friebertshäuser et al., 2010; Zhirnov et al., 2002; Zhirnov and Klenk, 2003), perhaps because proteases may be quickly inactivated and not accumulate in airway surface liquid (ASL) (Böttcher-Friebertshäuser et al., 2010; Zhirnov et al., 2002). An alternative paracrine HA cleavage by serum plasmin/plasminogen, which selectively docked to plasma membrane of infected cell (where plasminogen is converted into plasmin), was described for certain influenza strains in cultured cells (Goto and Kawaoka, 1998; Goto et al., 2001; Lazarowitz et al., 1973; Lebouder et al., 2010; Zhirnov et al., 1982a). In the intact host, this cleavage could occur only at the late stage of infectious process when there was transmigration of serum plasminogen or plasmin into infected tissues. The apical entry and release of influenza virus in respiratory tract make events on the basolateral surface less relevant to control of infection. The relatively high efficacy of cell-associated cleavage of monobasic influenza HA suggests that extracellular paracrine activation virus plays a minor role in influenza infection process in respiratory system. At the same time, it is worth of note that activation of certain influenza virus strains by plasmin is thought to play important role in brain tissue and specifically contribute to viral neurovirulence (Sun et al., 2010).

Another potential pathway of monobasic influenza virus proteolytic activation is a “cleavage from without” with cleavage occurring during virus entry into the target cell. In this pathway, a non-activated virion is bound to cell receptors through the HA0 protein and then activated by host membrane protease in the process of internalization at the plasma membrane or/and within the intracellular endosome in tissue cultures (Böttcher-Friebertshäuser et al., 2010; Boycott et al., 1994) and respiratory epithelium cells (Zhirnov et al., 2002).

The association of influenza infection with bacterial respiratory disease is well described. Notably, some bacteria produce proteases that activate infectivity of certain influenza viruses and enhance the development of influenza pneumonia in mice (Scheiblauer et al., 1992; Tashiro et al., 1987b). However, bacterial proteases seem to play only sporadic role in activation of certain influenza viruses (Callan et al., 1997; Scheiblauer et al., 1992) and mechanism of synergistic action of HA proteolytic activation and bacterial proteases in development of lung pathology remains to be fully defined (Akaike et al., 1989; Scheiblauer et al., 1992; Tashiro et al., 1987c).

Depending on whether a virus has mono- and multi-basic cleavage sites in their influenza virus HA proteins, there are two major classes of proteases that specifically recognize these sites. Predominantly, ubiquitous furin-like proteases of the subtilisin family were found to cleave the Arg in the multi-basic proteolytic site of highly virulent H5 and H7 influenza viruses (Garten and Klenk, 2009). Certain serine proteases of trypsin-like specificity appeared to cleave hemagglutinins of all human and avian virus subtypes (H1–H16) at the Arg in the monobasic cleavage site (Chen et al., 1998). The presence of such trypsin-like proteases activating human epidemic viruses with monobasic HA and permitting multicycle infection process was demonstrated in many host systems including: (1) cultured cells (human intestinal epithelium cell line Caco-2, human lung epithelium cell line Calu-3, Madin-Darby bovine kidney cells (MDBK), human hepatoma (line HepG2), swine kidney (line SPEV) and other cultured cells incubated with blood serum) (Böttcher-Friebertshäuser et al., 2010; Chaipan et al., 2009; Lazarowitz et al., 1973; Ollier et al., 2004; Zhirnov et al., 1982a; Zhirnov and Klenk, 2003), (2) chicken embryonated eggs (Zhirnov et al., 1985), (3) mouse heart, and lungs (Pan et al., 2011b; Zhirnov et al., 1984), and (4) human respiratory epithelium (Zhirnov et al., 2002, 2011). However, many cell lines lacking IAP, e.g. Madin-Darby canine kidney (MDCK), human alveolar type II (A549), baby hamster kidney (BHK-21), require the addition of exogenous trypsin for the HA cleavage and sustained multicycle replication of human influenza viruses.

Many proteases, such as tryptase Clara, plasmin, miniplasmin, kallikrein, human airway trypsin-like protease (HAT), epitheliasin (TMPRSS2; transmembrane protease, serine 2), transmembrane

protease, serine 4 (TMPRSS4) have been documented as potential influenza virus activating proteases (IAP) in model systems in cultured cells (Bertram et al., 2010; Böttcher et al., 2006; Böttcher-Friebertshäuser et al., 2010; Kido et al., 1992; Lazarowitz et al., 1973; Murakami et al., 2001; Sato et al., 2003; Towatari et al., 2002; Zhirnov et al., 1982a). However, which host protease(s) is most closely linked with influenza disease and responsible for the HA cleavage and activation of epidemic influenza viruses in human respiratory tract is not yet clear and remains an important research target.

3. Aprotinin targets influenza HA0 cleavage and suppresses multicycle virus replication

Aprotinin is the model protease inhibitor that has progressed the furthest in preclinical and clinical evaluation. One of the characteristics of aprotinin is its high general tolerance even at large dosages; for example, the LD50 in mice is 2.5×10^6 Kallikrein Inhibiting Units (KIU)/kg and in dogs intravenous administration of 1.0×10^6 KIU/kg is tolerated without complications (for review see (Trautschold et al., 1967)). It is a single-chain globular polypeptide isolated from bovine lung tissue. In bovine organs, it is synthesized as a longer precursor, which folds and then is cleaved into the mature 6.5 kDa polypeptide with an isoelectric point of 10.5 (Fritz and Wunderer, 1983). Mature bovine aprotinin consists of 58 amino acid residues folded into compact tertiary structure containing a twisted β-hairpin and a C-terminal α-helix and cross-linked by three intramolecular disulfide bridges (Cys5-Cys55, Cys14-Cys38 and Cys30-Cys51) (Fig. 2A and B). This structure provides remarkable stability of aprotinin and resistance to denaturation and proteolytic degradation by most known proteases. It does not lose activity in 2% trichloracetic acid, 70% methanol and ethanol, or 50% acetone (Kassell, 1970). Aprotinin is a highly conservative polypeptide among different animal species and only two (Arg17 → Lys17 and Ile18 → Met18) amino acids in bovine aprotinin are substituted for in the human analogue (Sun et al., 2009). A wide range of proteases including human plasmin, trypsin, kallikrein, chymotrypsin, activated protein C, thrombin, and neutrophil elastase are inhibited by aprotinin (Fritz and Wunderer, 1983). Lys-15 on the exposed loop of aprotinin binds tightly in the enzymatic pocket at the active serine site of target proteases and inhibits their proteolytic activity. Aprotinin rapidly and strongly interacts with many proteases, such as trypsin, chymotrypsin, plasmin, kallikrein. For example, dissociation constant for the aprotinin-trypsin complex is $K_i = 6 \times 10^{-14}$ mol/L, one of the lowest so far reported for a protein/protein interaction. However, aprotinin complexes less strongly with proteases, such as thrombin and elastase (Fritz and Wunderer, 1983; Trautschold et al., 1967). These variations may regulate physiologic effects of aprotinin in whole organism.

A number of years ago we postulated that influenza virus HA cleavage is an exploitable chemotherapeutic target (Zhirnov, 1983). To develop this idea, aprotinin was selected as the lead agent to suppress this virus-specific but host mediated event in infected model systems. The choice of aprotinin was based on its wide anti-protease spectrum and good physiologic tolerance in animals and humans (Fritz and Wunderer, 1983; Trautschold et al., 1967). It was demonstrated that aprotinin suppressed human influenza viruses by inhibiting HA cleavage and limiting resultant virus activation and multicycle infection in cultured cells (Böttcher-Friebertshäuser et al., 2010; Chaipan et al., 2009; Sato et al., 2003; Zhirnov et al., 1982a, 2002, 2011; Towatari et al., 2002; Zhirnov and Klenk, 2003), chicken embryonated eggs (Zhirnov et al., 1985), mouse lungs, and heart (Pan et al., 2011b; Zhirnov et al., 1984), and human respiratory epithelium (Zhirnov et al., 2002). Recently, it has been also observed, that aprotinin effectively inhibits hemagglutinin cleavage and replication of the pandemic 2009 H1N1 influenza virus, H1N1pdm, in many host systems including human respiratory epithelium (Zhirnov et al., 2011). These observations demonstrate that: (i) HA cleavage, as a virus specific target, is vulnerable to antiviral intervention and (ii) aprotinin has potential for therapeutic intervention against influenza in model systems and by implication in humans. This conclusion is strengthened by numerous observations that all of the above mentioned influenza-activating proteases (HAT, TMPRSS2, TMPRSS4, plasmin, miniplasmin, tryptase Clara, ectopic trypsin, etc.) are highly sensitive to aprotinin inhibition in cell culture experiments (Böttcher-Friebertshäuser et al., 2010; Kido et al., 1992, 1993; Murakami et al., 2001; Sato et al., 2003; Towatari et al., 2002; Zhirnov et al., 1982a,b,c).

After experiments in cultured cells and eggs, the therapeutic efficacy of aprotinin was studied by the Zhirnov group in animals infected with different influenza and parainfluenza viruses. It was found that this drug protected mice from lethal influenza A and parainfluenza virus infection and significantly reduced virus

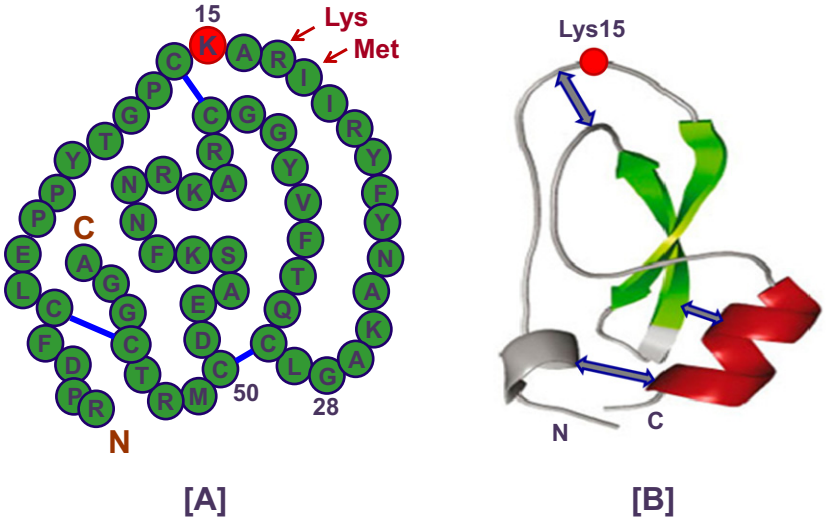

Fig. 2. Aprotinin structure. Cartoon representations of primary (A) and 3D (B) structures of bovine aprotinin were prepared according Helland et al. (1999). Mutations specific for human aprotinin are shown by red arrows in panel A. Lys 15 in the antiprotease active center is shown by red circle. β-sheet and α-helix structures are shown in tertiary structure in green and red, respectively. Disulfide bridges between Cys residues 5–55, 14–38, and 30–51 are indicated with double side arrows in blue.

titers and pathology in bronchi and lungs when administered either through intraperitoneal injections (Zhirnov et al., 1982a,b, 1984) or aerosol inhalations (Ovcharenko and Zhirnov, 1994). In these experiments, intraperitoneal injections of aprotinin were given for 5 days at 4–6 h intervals basing the knowledge that injected aprotinin distributed to most organs including lungs, bronchi, spleen, and pancreas with the individual organ deposition of 1–10% of the injected dose and that it was eliminated from them mainly by kidneys during 5 h (Werle et al., 1968). Inhalation therapy of mice included four 40 min exposures daily in aerosolized aprotinin flow for 5–6 days (Ovcharenko and Zhirnov, 1994). Administered inhalation and parenteral doses of aprotinin were estimated to be about 50 and 750 KIU/mouse/day, respectively (Ovcharenko and Zhirnov, 1994; Zhirnov et al., 1982a,b).

In this set of experiments mouse-adapted influenza A viruses possessing the HA with monobasic cleavage site were used as challenge viruses. Aprotinin injections were found to suppress influenza virus activation and replication through the blockage of the HA0 cleavage in lungs of infected mice (Zhirnov et al., 1984). It was also revealed in chicken model that parenteral injections of aprotinin decreased virus dissemination from primary infection locus throughout the body of influenza infected birds (Zhirnov et al., 1982b). Similar data that low molecular weight protease inhibitors, such as aprotinin (mol. wt. 6.5 kDa), leupeptin (0.43 kDa), and camostat (0.5 kDa), were able to suppress replication of influenza and parainfluenza viruses and development of pneumonia and viral myocarditis in mice were obtained by others (Hayashi et al., 1991; Hosoya et al., 1992; Pan et al., 2011b; Tashiro et al., 1987a). The *in vivo* data supported our concept of treatment of influenza by aprotinin or other protease inhibitors.

In vivo experiments with highly pathogenic viruses characterized by a multibasic HA cleavage site were also performed using an avian laboratory model. Delay in the time to death and reduced viraemia were observed in chickens infected with highly pathogenic virus A/tern/South Africa/63 (H5N3) after intraperitoneal injections of aprotinin (Zhirnov et al., 1982b). We postulate that the defined but lower therapeutic effect against the pathogenic H5N3 virus in chickens was mainly provided by anti-inflammatory effect of aprotinin and that aprotinin may not efficiently inhibit multibasic HA cleavage perhaps because of insufficient concentrations at the intracellular site at which multibasic cleavage occurs. Effective suppression of replication of viruses with multibasic HA is potentially possible by means of modifying the active center of aprotinin or adding intracellular targeting signals, such as hydrophobic anchors or additional signal peptides. Such modifications of aprotinin have been proposed earlier to change its enzymatic specificity (Krowarsch et al., 2005). An intracellularly-targeted chimeric aprotinin has been designed in our laboratory. This chimeric aprotinin molecule containing additional signal peptides was prepared by recombinant technology using site-directed mutagenesis and was expressed in mammalian cells via the DNA expressible construct. Encouragingly, it has displayed promising intracellular antiviral effect against H5N1 virus HA0 cleavage (Zhirnov, studies in progress).

Virus activation through HA cleavage by host proteases and the therapeutic effect of aprotinin suggest that these events play a key role in disease pathogenesis. Influenza infection in the human respiratory tract seems to amplify via a "vicious cycle" in which virus infection of epithelial cells stimulates host proteases which in turn trigger proinflammatory proteolytic cascades with resultant further activation of progeny virus to enhance multicycle growth (Zhirnov, 1987). This concept concerning virus–host proteases interplay is illustrated in Fig. 3. Influenza virus is known to stimulate host cell proteases which trigger proinflammatory reactions through the activation of plasmin-, kallikrein-, thrombin-, and trypsin-dependent cascades in respiratory and myocardial systems (Akaike et al., 1989, 1996, 1990; Barnett et al., 1990; Hennet et al., 1992; Lazarowitz et al., 1973; Pan et al., 2011b; Towatari et al., 2002). Natural protease inhibitors, which normally maintain proteolytic balance in respiratory system, are down-regulated in infectious loci (Akaike et al., 1989; Beppu et al., 1997; Heimburger, 1975; Hennet et al., 1992; Reichert et al., 1971). This antiprotease deficit and protease overbalance may additionally aggravate influenza pathogenesis. In our model, exogenous aprotinin or similar protease inhibitors may be able both to suppress multicycle virus replication and attenuate pathology and inflammation, as illustrated in Fig. 3.

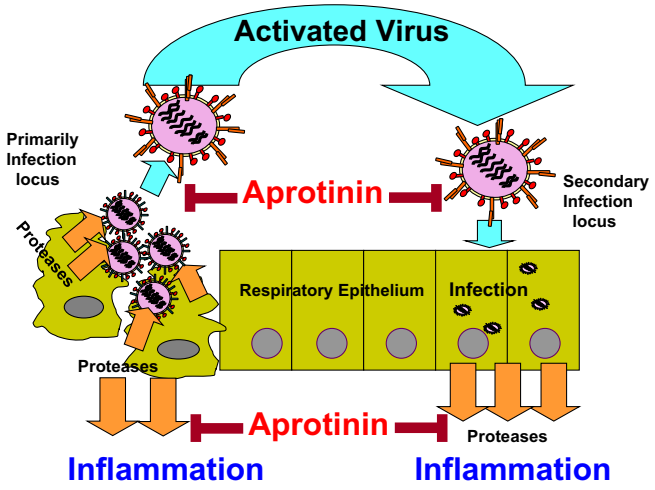

Fig. 3. "Vicious cycle" in influenza pathogenesis and aprotinin targets. Proteolytic events during influenza virus infection develop by the mechanism of "vicious cycle". Virus stimulates host proteases, which is one of the key factors of influenza pathogenesis (see Table 1). Up-regulated host proteases activate fusogenic activity and infectivity of synthesized virus through the cleavage of HA with promotion of infection spread. Virus-induced cell damage provokes initial inflammation that induces a leakage of plasmin, kallikrein, thrombin and transcytosis of leucocytes from blood stream into tissues causing a secondary superstimulation of inflammatory process. Aprotinin, suppressing host proteases, decreases spread of virus infection and development of inflammation to provide therapeutic effect.

4. Benefits and precautions in aprotinin usage against influenza

The preclinical efficacy of aprotinin against influenza *in vitro* and *in vivo* experiments prompted us to study aprotinin therapy in humans with influenza and influenza-like infections. Since influenza infection primarily locates in the upper-middle respiratory tract of humans, small-particle aerosol inhalations were utilized. A clinical study was conducted during a seasonal outbreak of acute respiratory illness caused predominantly by influenza and parainfluenza viruses (January–May 1994) (Zhirnov et al., 1996a,b). Interested readers who are unable to access these reports may contact the corresponding author to obtain copies. Eighty-four patients 14–68 years of age were admitted to the Infectious Clinics with acute febrile status less than 2 days duration. Influenza clinical diagnosis was confirmed by direct immune fluorescent antibody test of cells from nasal–pharyngeal swabs. Aprotinin was dissolved in sterile water and aerosolized through stationary ejector nebulizer (Aerosol Inhaler Model 1; Respirator Ltd., Moscow, Russia) generating aerosol particles with a mass medium diameter 2–10 μm. In the aerosol cloud delivered to the patients about 30–50% of these particles aggregated leading to diameters up to 50–100 μm.

The therapeutic course consisted of three six-min inhalations daily beginning on day 2 from the onset of clinical symptoms. The received aprotinin inhalation dose was about 1500–

2000 KIU/person/day. Therapeutic efficacy as indicated by about twofold reduction in duration of fever, headache, throat pain, hoarseness, cough, and earlier discharge from the hospital, Fig. 4. Thus a clinical trial conducted by double-blind and randomized design documented efficacy of aprotinin aerosol inhalations in patients with seasonal influenza and parainfluenza. These observations are consistent with preclinical data showing an effectiveness of aerosolized aprotinin in mice infected with influenza or parainfluenza type 1 viruses possessing similar monobasic activation cleavage sites (Ovcharenko and Zhirnov, 1994). Importantly, aprotinin inhalations were well tolerated by patients and no adverse reactions were observed in any patients treated with aerosol aprotinin (Zhirnov et al., 1996a,b). Similarly good tolerance and a marked therapeutic effect were documented in patients with chronic obstructive pulmonary disease (COPD) treated with aprotinin inhalations (Rasche et al., 1975).

The above clinical data are in agreement with our preclinical toxicity tests of aerosolized aprotinin in animals. Parameters of general toxicity such as biochemical and coagulation indices and cellular composition of blood, histological examinations of lungs, bronchi, hart, liver and kidneys, as well as CNS and hart functionalities were tested in rabbits and rats given 20-min aprotinin inhalations daily for prolong period, 30 days (Zhirnov et al., 1994a). Local irritant actions of aprotinin were sought by eye conjunctiva probe and active anaphylaxis and delayed type of hypersensitivity were evaluated by dermal probes in guinea pigs sensitized with 30-day long course of 10-min daily inhalations of aerosolized aprotinin (Zhirnov et al., 1994b). All preclinical tests demonstrated a lack of allergic and irritant reactions and systemic toxicity of aerosolized aprotinin (Zhirnov et al., 1994a,b). Experiments in various cell cultures infected with influenza A viruses proved low toxicity of aprotinin and showed that the aprotinin therapeutic index was greater than 200 (Zhirnov et al., 2011).

The therapeutic efficacy of aprotinin against influenza may be mediated by two major actions. The first one is antiviral activity of aprotinin as described above. The second proposed action is anti-inflammatory activity of aprotinin which may additionally diminish pathology at the site of infection and contribute to more rapid resolution of disease. The anti-inflammatory action of aprotinin is polyvalent with its main targets summarized in Table 1. Aprotinin, through inhibition of multiple mediators (e.g., kallikrein, plasmin, thrombin, oxide radicals, and protease-activating receptors) may attenuate inflammatory pulmonary responses, such as leakage of plasmin, kallikrein, thrombin, transmigration of monocytes into lung tissue, edema, tissue oxidative stress, and disturbed mucociliary clearance. These inflammatory factors are known to be components of the pathogenesis of influenza disease, including influenza pneumonia and myocarditis, both in animals and humans (Akaike et al., 1989, 1996, 1990; Barnett et al., 1990; Hennet et al., 1992; Pan et al., 2011b). Recent observations obtained in cell culture and in mice infected with influenza virus supported this concept. Aprotinin was shown to suppress both virus replication and influenza-induced up-regulation of proinflammatory cytokines, such as IL-6, IL-1b, TNF-alpha, matrix metalloproteases in cultures of cardiomyocytes and in the myocardium of infected mice (Pan et al., 2011a,b). Thus, administration of aprotinin may be beneficial for influenza patients because it limits infection and attenuates pathological lesions.

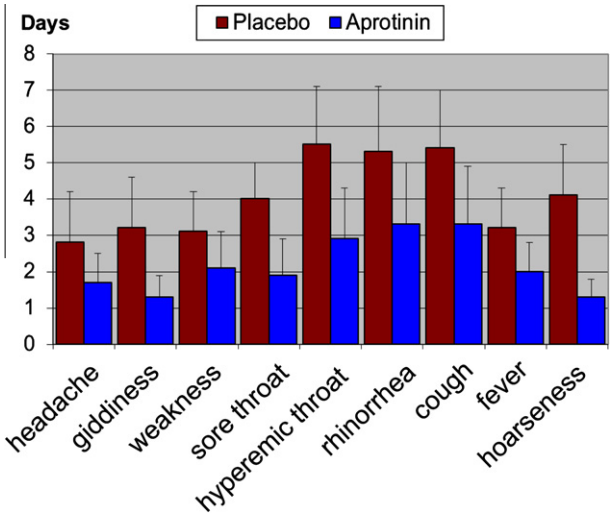

Fig. 4. Duration of clinical symptoms in influenza patients treated with aerosolized aprotinin. A clinical study was conducted during a seasonal outbreak of Influenza in Moscow winter-spring season 1994 (Zhirnov et al., 1996a,b). Patients were given three six-min inhalations daily for 4–5 days. Placebo patients received alkaline aerosol inhalations generated from 0.6% solution of sodium bicarbonate without aprotinin. Mean duration (±SD) of symptoms was determined from the beginning of the aprotinin treatment to the time when the symptom disappeared. Shortening of symptoms duration in aprotinin-treated vs. placebo patients was statistically significant for all symptoms ($p < 0.05$).

Thus, two approaches to therapy of influenza with aprotinin can be considered. The first is in mild-to-moderate clinical influenza when influenza is predominantly in the upper respiratory – nasal–pharyngeal and trachea-bronchial areas. In this case, aerosol aprotinin can be delivered through an oral or nasal approach. On the basis of our clinical trials (Zhirnov et al., 1996a,b), for the treatment of influenza a minimal-dose of $1–3 \times 10^3$ KIU of aerosolized aprotinin per day per person can be considered. A pocket meter dose inhaler (MDI) containing aprotinin is a convenient devise which provides individual use that is important to prevent nosocomial cross-contamination between patients. A metered dose pocket inhaler generating aerosolized aprotinin (85 KIU per one inhalation dose) was designed on the basis of ozone-saving technology (Modulite™ (Ganderton et al., 2002)) and has been licensed recently for human use in Russia (under the trade mark Aerus™; Certificate 000280/10 dated 25-01-2010) as anti-influenza drug for treatment of seasonal and swine-origin pandemic flu.

A second approach of aprotinin therapy may be a systemic intravenous injection. This is theoretical but is based on the licensed intravenous use of this drug for other indications. It is known that after intravenous injection, rapid distribution of aprotinin occurs into the total extracellular space and up to 10% of injected dose is deposited in bronchial-lung tissues (Kaller et al., 1978; Werle et al., 1968). With such deposition, a dose regimen as low as $0.3–5.0 \times 10^5$ KIU of aprotinin/day infused intravenously might be enough to provide local therapeutic effect in the respiratory tract against severe influenza. However, to recommend an exactly adequate therapeutic dose, the local inhibitory activities of aprotinin in the airway and in plasma remain to be investigated with differing aprotinin dosing regimens. Similarly, effective therapeutic doses of intravenous or intraperitoneal injections of aprotinin to produce sufficient local inhibitory activity against acute severe pancreatitis are still being debated (Seta et al., 2004; Smith et al., 2010). For life threatening moderate-to-severe influenza infection (Fujimoto et al., 1998; Lyon et al., 2010) and highly pathogenic avian H5N1 for which the mortality exceeds 50% (Beigel et al., 2005), intravenous aprotinin is worth assessing in hopes that aprotinin might inhibit pulmonary and systemic virus replication and also reduce inflammation and resultant cytokine response systemically and in the respiratory tract. Clearly, further clinical studies are needed to evaluate this infusion use of aprotinin and optimize dosage regimens.

Aprotinin and similar antiproteases inhibiting influenza virus HA0 cleavage and preventing virus activation can be used in combination with chemotherapeutic agents directed against other

Table 1
Anti-inflammatory action of aprotinin.

Aprotinin targets	Anti-inflammatory effects of aprotinin	References
1. Plasmin	a. Suppresses fibrinolysis and, prevents hemorrhage but does not prevent dissolution of blood clots in small vessels decreasing blood stasis	(Ray and Marsh 1997) (Wachtfogel et al., 1993)
	b. Suppresses kallikrein cleavage and super production of bradykinin	(Attali et al., 2008)
	c. Inhibits plasmin-dependent transmigration of bacteria across respiratory epithelium	(Akaike et al., 1989)
	d. Down regulates plasmin-induced proinflammatory activation during infectious pneumonia	(Passero et al., 2010)
	e. Normalizes sodium homeostasis by inhibiting plasmin-dependent activation of extracellular Na channel (ENaC).	
2. Kallikrein	a. Suppresses proinflammatory cytokine production	(Poullis et al., 2000) (Wachtfogel et al., 1993; Wachtfogel et al., 1995)
	b. Prevents kininogen cleavage and decreases generation of kinins	
	c. Preserves capillary permeability	(Yayama et al., 2003)
	d. Attenuates early fibrinolytic events	
	e. Attenuates kinin-dependent extravasation (transplasmosis) of serum proteases	
3. Monocyte and granulocyte adhesion molecules	a. Prevents the expression of pro-inflammatory adhesive integrins (e.g., CD11b) and decreases neutrophil degranulation and extravasation at inflammatory sites	(Harig et al., 1999) (Gilliland et al., 1999)
	b. Down regulates release of TNF-α, IL-6, IL-8, elastase	(Asimakopoulos et al., 2001)
	c. Elevates secretion of anti-inflammatory IL-10	(Pruefer et al., 2003)
4. Nitric oxide synthase (NOS)	Reduces concentration of oxide radicals (NO, O_2^-) and their deleterious effects on tissues at infection locus	(Bruda et al., 1998) (Hill and Robbins, 1997)
5. Prostasin/epithelial sodium channel (ENaC)	a. Suppresses activation of protease-activated ENaC in respiratory epithelium and normalizes mucus viscosity and water balance in airway surface fluid (ASL)	(Planès and Caughey, 2007) (Coote et al., 2009)
	b. Enhances mucociliary clearance	
6. Complement components 3a, 5a	a. Attenuates plasmin-dependent complement generation	(Huber-Lang et al., 2006)
	b. Decreases activation of complement due to reduced formation of C3a and C5a	(Amara et al., 2008) (Sarma et al., 2006)
7. Platelet protease-activated receptor (PAR-1)	a. Prevents thrombin-induced platelet dysfunction and over aggregation and decreases vascular thrombosis	(Khan et al., 2005) (Poullis et al., 2000)
	b. Preserves platelet glycoprotein receptor (GPIa, GPIIb/IIIa) function	

viral and/or host targets. These combinations will have two major benefits. First, combination of drugs targeting different virus-specific functions will potentially increase virus inhibition and decrease a risk of emergence of drug-resistant virus mutants. Second, drug combinations may elevate therapeutic efficacy at lower dosages of agents diminishing their adverse complications. It was shown earlier that intraperitoneal injections of aprotinin combined with rimantadine markedly increased protection, in comparison to each drug alone, of mice lethally infected with mouse-adapted influenza A/Aichi/2/68 (H3N2) virus (Zhirnov et al., 1985). To enhance effectiveness against severe influenza, intravenous aprotinin can be complemented with parenteral forms of peramivir, ribavirin, amantadine, or their improved derivatives targeting different virus functions as well as with anti-inflammatory and anti-apoptotic agents (celecoxib, mesalazine (Zheng et al., 2008)) and antioxidants (N-acetylcysteine (Garozzo et al., 2007; Ghezzi and Ungheri, 2004)) interfering with host mechanisms.

For aerosol administration, development of metered dose manual inhaler containing multivalent combination of compounds applicable in aerosolized forms, such as protease inhibitor (a blocker of the HA0 cleavage), zanamivir (an inhibitor of virus NA) (Moscona, 2005), sialidase (DAS181) (an inactivator of virus receptors on target cells) (Malakhov et al., 2006; Zhang, 2008), ribavirin or T-705 (favipiravir) (suppressors of virus polymerase function (Eriksson et al., 1977; Furuta et al., 2005)), and celecoxib (anti-inflammatory agent) (Patlolla et al., 2011) might be promising for effective influenza therapy and use in general medical practice during influenza outbreak.

Any proposed use of aprotinin against influenza must acknowledge several precautions. In spite of relative tolerance (Fritz and Wunderer, 1983; Trautschold et al., 1967) it has been shown in the high doses used in patients undergoing high-risk cardiac surgery to have a greater risk of post-operative death than lysine analogue antifibrinolytic agents (Fergusson et al., 2008). The complications of concern in observational studies of aprotinin were renal, cardiac, or cerebral events at high-dose regimen (loading dose, 2 million kallikrein-inhibitor units (KIU); and intravenous infusion of 500,000 KIU per hour of surgical operation) used in cardiac surgery (Mangano et al., 2006; Shaw et al., 2008). The importance of the deleterious effects of high doses of aprotinin continue to be debated (Hogue and London, 2006; McEvoy et al., 2007; Pasquali et al., 2010). These risks were mentioned to be insignificant with a two times lower intravenous doses (Mangano et al., 2006). It should be emphasized that aprotinin doses proposed for aerosol and intravenous administration in influenza therapy are about 1000 and 30 times, respectively, lower than those ones usually used in cardio-thoracic surgery.

Because aprotinin is a compound isolated from bovine lungs, it bears a risk of sensitization of the recipient with hypersensitivity and anaphylactic reactions with repeated use. In clinical intravenous administrations of aprotinin, such allergic reactions were reported to be rare (<0.1%) in patients with no prior exposure to aprotinin but the drug might be used repeatedly in influenza. The risk for such adverse reactions is expected to be increased up to 2.7% in reexposed patients and must be weighed against the benefit of aprotinin to patient (Beierlein et al., 2005; Levy and Adkinson, 2008). Very rare allergy to aprotinin may be life threatening (Vusicevic and Suskevic, 1997). Notably, this allergic risk was reported with high intravenous doses of aprotinin, $3–7 \times 10^6$ KIU per day. Whether or not it will develop in patients received significantly lower aerosol doses is not known. In this regard, it is important to emphasize that fibrin sealant containing aprotinin is widely applied onto mucus surfaces in surgical patients (Busuttil, 2003). In this clinical practice, hypersensitivity and allergic reactions were extremely rare (only five reports following 1 million exposures to fibrin–aprotinin sealants (Beierlein et al., 2000)). Nevertheless, such adverse reactions must be kept in mind as a precaution of aprotinin usage. In our clinical trials of aerosolized aprotinin inhalations against influenza (about 300 patients received such inhalations), the lack of any allergic and irritant adverse reactions was documented (Zhirnov et al., 1996a; Zhirnov, unpublished data). In the near future, human recombinant aprotinin, which can be generated by biotechnological approach (Meta et al., 2009;

Sun et al., 2009), may be used in influenza patients to mitigate these hypersensitivity risks.

5. General conclusions

Host trypsin-like proteases are key factors in facilitating influenza virus infection because they cleave virus HA glycoprotein to trigger its cell fusogenic activity and activate virus infectivity with promotion of infection. In turn, influenza virus directly damages airway epithelium and stimulates host proteases, which mediate development of inflammation in the infection locus. Aprotinin, a polyvalent protease inhibitor from bovine lung, was found to suppress influenza virus proteolytic activation and spread of infection and to attenuate pathology and inflammation and these effects resulted in achieved therapeutic effect. It is recommended that aerosol inhalations and intravenous infusion of aprotinin and other proteases inhibitors be further explored for the treatment of mild-to-moderate and severe forms of influenza, respectively. Aprotinin and other antiproteases inhibiting influenza virus HA cleavage can potentially be used in combination with chemotherapeutic agents directed against other viral and host targets in the treatment of severe influenza.

Disclosure statement

The funders had no role in study design, data analysis, decision to publish, or preparation of the manuscript. OPZ has US, European, Japan, and Russian patents disclosing the use of aerosolized aprotinin for the treatment of influenza. Other authors declare no competing interests.

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