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Airway proteases: an emerging drug target for influenza and other respiratory virus infections

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To enter into airway epithelial cells, influenza, parainfluenza- and coronaviruses rely on host cell proteases for activation of the viral protein involved in membrane fusion. One protease, transmembrane protease serine 2 (TMPRSS2) was recently proven to be crucial for hemagglutinin cleavage of some human influenza viruses. Since the catalytic sites of the diverse serine proteases linked to influenza, parainfluenza- and coronavirus activation are structurally similar, active site inhibitors of these airway proteases could have broad therapeutic applicability against multiple respiratory viruses. Alternatively, superior selectivity could be achieved with allosteric inhibitors of TMPRSS2 or another critical protease. Though still in its infancy, airway protease inhibition represents an attractive host-cell targeting approach to combat respiratory viruses such as influenza.

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Introduction

On a global scale, seasonal influenza A and B viruses cause around 3–5 million cases of severe illness each year resulting in about 250 000–500 000 deaths [1]. The existing drugs directing the influenza neuraminidase (oseltamivir, zanamivir, peramivir and laninamivir) or M2 proton channel (amantadine and rimantadine), show rather modest clinical efficacy and established or potential resistance. Besides, pandemic variants may at any time emerge due to gene reassortment between human and zoonotic influenza viruses and rapid virus spread in a globalized society. Novel medications with an inventive mode of action are urgently needed to treat or prevent severe influenza infections in vulnerable populations [2]. A recent WHO BRaVe (Research needs for the Battle against Respiratory Viruses) [3] report calls for host cell targeting antiviral approaches, since these are anticipated to exhibit a higher barrier for drug resistance. We here focus on a new paradigm to combat influenza or other respiratory virus infections, which is directed towards airway proteases with an essential role in virus activation. Complementary to our concise analysis, some detailed reviews were recently published elsewhere [4,5•,6–8].

The influenza hemagglutinin as antiviral target

The influenza virus hemagglutinin (HA) mediates two events in virus entry: (i) the HA globular head binds to sialylated cell surface glycans resulting in virus endocytosis; and (ii) the HA stem refolds at endosomal pH to liberate the fusion peptide, causing fusion of the viral and endosomal membranes and release of the viral genome into the host cell cytoplasm. Hence, HA targeting entry blockers may (i) interact with the HA receptor binding site to inhibit virus attachment; or (ii) bind to the HA stem region and inhibit fusion, for instance by preventing its refolding at acidic pH. Design of broad influenza HA blockers is anything but easy, given the high sequence variability among HA subtypes; antigenic drift of HA; different receptor usage of avian versus human influenza viruses; and multivalent nature of the HA-receptor interaction. For instance, small molecule influenza fusion inhibitors appear inadequate since they generally show subtype specificity and rapid emergence of resistance [9].

An alternative and indirect strategy is to prevent the proteolytic activation of HA by inhibiting the host cell proteases responsible thereof. In influenza virus-infected cells, HA is synthesized as the precursor protein HA0 (~75 kDa), which assembles into a noncovalently linked homotrimer. To obtain fusion capacity, HA0 needs to be cleaved, by a cellular protease, into HA1 (~50 kDa) and HA2 (~25 kDa). This enables insertion of the fusion peptide (now located at the N-terminus of HA2) into a negatively charged cavity, priming the HA for low pH-dependent fusion [7].

Highly pathogenic avian influenza A viruses (HPAIVs) of subtypes H5 and H7 possess a multibasic cleavage site that is cleaved by ubiquitously expressed proteases, explaining the systemic infections of these viruses in avian species [7]. The multibasic cleavage site R-X-K/R-R, with Arg at position P4, is processed in the trans-Golgi network by furin and proprotein convertase (PC) 5/6 [7], calcium-dependent serine proteases of the subtilisin

superfamily which have a different fold yet the same catalytic triad (Asp–His–Ser) as trypsin-like enzymes [10]. For HPAIVs possessing a K-K/R-X-R motif, the P4-Lys significantly suppresses cleavage efficiency by furin and PC 5/6. These HAs are activated by type II transmembrane serine proteases (TTSPs), more specifically mosaic serine protease large-form (MSPL) or its splice variant TMPRSS13 [11].

In contrast, the HA0 proteins of human influenza A and B viruses possess a cleavage loop with a monobasic (*i.e.* single arginine) cleavage site. This feature is also present in pandemic influenza A viruses, whether of H1N1 (including the viruses of 1918 and 2009), H2N2 or H3N2 subtype. Inhibition of the HA-cleaving host cell proteases induces production of non-infectious virions, thus halting further virus replication. This type of inhibitors could have two major strengths: (i) resistance is less likely to develop since a host protein is targeted instead of a highly mutable viral protein; and (ii) broad activity against diverse respiratory viruses may be envisaged since, besides influenza virus, several other respiratory viruses rely on activation by similar proteases (see below). Hence, these drugs might also be used to tackle outbreaks by respiratory viruses for which no specific antiviral drugs are available.

Host proteases involved in HA0 cleavage activation

As summarized in Table 1, diverse trypsin-like proteases (TLPs) are able to activate human influenza viruses *in vitro* or *in vivo*. During virus propagation in chicken eggs, HA0 cleavage is performed by blood clotting factor Xa in the allantoic fluid [12], while in Madin Darby canine kidney (MDCK) cell cultures, addition of trypsin is needed for multicycle replication [13]. As for HA activation in the airways of infected persons, several TLP candidate enzymes have been identified, but the picture is still far from complete. A role is now well established for transmembrane protease serine 2 (TMPRSS2), a member of the TTSP family, which are integral membrane proteins with an extracellular C-terminal serine protease domain and an N-terminal cytoplasmic domain. TMPRSS2-knockout (KO) mice were found to survive infection with an H1N1 or H7N9 influenza virus that was lethal in wild-type (WT) mice [14••,15,16]. Furthermore, a genome wide association study showed that patients with higher TMPRSS2 expression levels had higher risk for severe infections by the 2009 pandemic H1N1 virus and increased susceptibility to influenza H7N9 [17••]. For the other circulating human influenza A subtype, H3N2, the protease profile is less well defined and appears to be strain-dependent. Compared to WT, TMPRSS2-KO mice proved equally [15] or slightly less vulnerable [14••] to lethal H3N2 infection. A third H3N2 strain was avirulent in TMPRSS2-KO mice but became lethal after ten passages in mice [16,18]. Since the passaged virus carried an *N*-glycosylation mutation at the bottom of the HA stalk region, the loss of this glycan may alter the accessibility of the cleavage loop and provide access to an alternative host protease [18].

A second TLP candidate is the closely related TMPRSS4 enzyme [19,20]. When infected with an influenza H3N2 strain, TMPRSS2/TMPRSS4 double-KO mice displayed lower morbidity and mortality, though showing some body weight loss, pathology and processing of HA [21]. Thus, besides TMPRSS2 and TMPRSS4, additional proteases are able to cleave and activate the HA of H3N2 viruses. A possible explanation is provided by a conformational difference seen in available HA0 crystal structures [22,23]. In H3 HA0, the cleavage loop extends from the protein surface, whereas in H1 HA0, the cleavage site is less exposed. Hence, H3 HA0 may be more accessible to diverse proteolytic enzymes [21]. This might allow for more efficient replication of the H3N2 virus, possibly explaining why the symptoms of H3N2 infections tend to be more severe compared to those of the H1N1 subtype [24].

A role for human airway trypsin-like protease (HAT) was proposed several years ago [25••] but still awaits *in vivo* verification in the existing and viable HAT-KO mouse model [26]. A systematic analysis of the HA0 cleavage pattern for 16 influenza A HA subtypes, was performed in cell assays using coexpression of HA0 plus TMPRSS2 or HAT protease [27•]. Under these testing conditions, cleavage by TMPRSS2 seemed, overall, more efficient than that by HAT, although marked subtype dependence was seen and neither of the two proteases was able of activating all 16 HA subtypes. Analysis of the cleavage site sequences of these 16 HAs did not point to any amino acid as being an absolute determinant for recognition by TMPRSS2 or HAT [27•].

Besides TMPRSS2, TMPRSS4 and HAT, other members of the TTSP family, namely matriptase [28–30], DESC1 (differentially expressed in squamous cell carcinoma gene 1) [31], MSPL (mosaic serine protease large-form) and its splice variant TMPRSS13 [11,31] can mediate cell membrane-associated HA0 cleavage of certain HA subtypes. These investigations were performed in HA0-expressing cells which were exposed to recombinant protease or engineered to overexpress the protease under study. The effect of matriptase was further demonstrated in Calu-3 cells (a cell line derived from human bronchial epithelia), in which matriptase gene knockdown resulted in significant impairment of influenza H1N1 replication [28]. *In vivo* investigations are hindered by the fact that matriptase-KO mice are non-viable [32].

Additionally, secreted proteases like plasmin [33,34] and kallikrein (KLK) types 5 and 12 [35] can perform H1 and H3 HA0 cleavage [35]. Although all these proteases are