Influenza Virus H1N1 Inhibition by Serine Protease Inhibitor (Serpin) Antithrombin III

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Abstract— Endogenous serine protease inhibitors (serpins) are anti-inflammatory mediators with multiple biologic functions. Serpins are also part of the early innate immune response to viral infection that includes mannose binding lectins, soluble CD14, defensins and antimicrobial peptides. Recently, serpin antithrombin III (ATIII) was shown to have broad-spectrum antiviral activity against HIV, HSV and HCV. We tested ATIII’s antiviral activity against a variety of influenza virus strains. In our studies we found strong in vitro inhibition of influenza virus A H1N1 isolates. Our data also demonstrate that ATIII potency was more than 100-fold that of ribavirin. We also found that inhibition was dependent on viral hemagglutinin with decreasing efficacy in the order of H1N1 > H3N2 > H5N1

>> Flu B. In vivo efficacy is currently still lacking demonstrating need for more advanced delivery methods for this biomolecule. Understanding how ATIII regulates influenza virus inhibition may reveal new avenues for therapeutic interventions.

Keywords — Serpin; Antithrombin III; influenza virus H1N1; hemagglutinin dependent.

I. INTRODUCTION

Approximately 500 million people are infected by influenza virus infection each year with annual epidemics producing significant morbidity and mortality. Influenza virus infection is usually a self-limiting disease among healthy adults, but can also lead to severe secondary bacterial pneumonia in elderly individuals and infants. The possibility exists that a highly virulent strain of influenza virus, such as the H5N1 avian influenza strain which emerged originally in Hong Kong in 1997 [1] or the ‘Spanish flu’ outbreak of 1918 [2], may adapt to become easily transmissible among humans and cause serious pandemics. At the present time, options for controlling influenza infection are limited. Protection through vaccination is limited due to the antigenic variation of the influenza virus. Currently four antiviral therapies have been approved for the prevention and treatment of influenza virus infection. The drugs amantadine and rimantadine, which inhibit influenza A as M2 ion channel blockers, are limited by adverse side effects, their lack of effectiveness against influenza B viruses, and the rapid development of viral resistance (>90% in some studies) [3]. Inhibitors of neuraminidase (NA), zanamivir and oseltamivir, are able to reduce virus replication of both influenza A and B but this class of inhibitor is also limited by the rapid development of viral resistance. The continued development of new agents with enhanced potency, reduced toxicity, and a greater genetic barrier to resistances, as well as targeting other steps in the influenza replication cycle is a critical need for the effectiveness of influenza therapy. As is the case with HIV, the development of highly active combination therapeutic strategies, perhaps used in parallel with available prophylactic measures, will yield an optimal strategy for controlling influenza virus and it is imperative that these strategies be in place prior to the appearance and spread of any pandemic human strain which is expected to result from cross-species transmission from the avian and swine influenza populations.

The course and out-come of an influenza virus infection is influenced by viral and host factors. Host risk factors, like obesity or pregnancy, became evident during the recent swine flue pandemics [4]. Furthermore, genetic factors in humans associated with higher susceptibility to influenza infections and severe disease outcome have been suspected for the 1918 pandemics, as well as the H5N1 human infections [5-9]. Multiple serine protease activities in the lung are also implicated in mediating influenza infection [10]. Therefore, blocking damage of influenza virus activated proteases in lung epithelia by the use of serine protease inhibitors may provide an alternative approach for treatment of influenza infection. Furthermore, host serine proteases are essential for the influenza virus life cycle because the viral haemagglutinin (HA) is synthesized as a precursor that requires proteolytic maturation.

We have developed the serine protease inhibitor (serpin) antithrombin III (ATIII) as a potential broad-spectrum viral inhibitor [11-15]. This protein belongs to a group of serpins which are part of the early physiologic response to viral infection that includes mannose binding lectins, soluble CD14, defensins, antimicrobial peptides, neutrophils, monocyte/macrophage and natural killer cells [16]. We feel that this molecule is well suited since it has an unusual long 72 h half-life in lungs that is longer than that of current small molecule inhibitors. Together, this longer half-life and the induction of an intrinsic anti-viral host-cell defense typical for ATIII [11, 13, 14] might prevent the occurrence of drug...
resistant influenza strains.

II. MATERIAL AND METHODS

Ethical statements

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University. The work was done in the AAALAC-accredited Laboratory Animal Research Center of Utah State University. Initial accreditation was granted on Feb 10, 1986 and has been maintained to the present time (last renewal: March 31, 2010). The Animal Welfare Assurance Number is A3801-01 and was last reviewed by the National institutes of Health on April 7, 2010 in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2002 Edition) and expires on February 28, 2014.

Source of ATIII

For our studies we used cGMP produced ATIII which was more than 95% pure as tested by C4 HPLC or by coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels. Serum derived ATIII (Talecris, Durham, NC) was used for the in vitro studies. For the in vivo efficacy studies we used human recombinant ATIII produced by GTC Biotherapeutics (Framingham, MA) in transgenic goats.

In vitro human influenza virus inhibition assays

We measured viral inhibition by ATIII with the following methods: 1) inhibition of virus-induced cytopathic effect (CPE), as determined by visual (microscopic) examination of the cells, 2) increases in the uptake of the vital dye neutral red (NR) into cells (NR Assay), and 3) virus yield reduction (Virus Yield Assay) as determined by endpoint dilution titration in MDCK cells. All tests have been previously described [17]. CPE inhibition data were expressed as the 50% effective concentration (EC50); toxicity was measured by determining the 50% cytotoxic (cell-viability) concentration (IC50) in stationary cell monolayers; and the selectivity index (SI) was determined as IC50/EC50. Eight one-half log10 concentrations of the test compounds were evaluated in triplicate wells containing influenza virus-infected MDCK cells. Standard placebo-treated virus controls, toxicity controls, ribavirin and normal-medium controls were included in all assays.

In vivo efficacy studies for ATIII using mouse-adapted H1N1

We tested the in vivo efficacy of ATIII using an influenza A/NWS/33-m (= mouse adapted) (H1N1) virus infection in mice, and compared it with ribavirin. ATIII III (400 µg, 200 µg, 100 µg/mouse) in 50 µl was administered intranasally once a day for 5 days starting 2 hours prior to virus challenge. 75 mg/kg (300 µM/kg) ribavirin was tested as a control with twice daily intraperitoneal (i.p.) administrations. Influenza A/NWS/33-m (H1N1) virus was administered intranasally at approximately 5 x 103 pfu (~3 x mLDS0).

Statistical analysis

Survival curves for in vivo evaluations were initially compared by the Mantel-Cox log-rank test, and statistical significance was found. Subsequently, pairwise comparisons of survival curves were made using the Gehan-Breslow-Wilcoxon test with Bonferroni corrected threshold of significance for the number of treatment groups evaluated. Calculations were made using the Prism 5.0 software program (GraphPad Software, San Diego, CA).

III. Results

In vitro human Influenza virus inhibition by ATIII

We found that ATIII was most active for Flu A (H1N1) viruses with yielded EC50 values ranging from 30 to 150 nM (Tab. I) and was most active for influenza A H1N1 isolates California/07/2009 and New Caledonia/20/99. We did not detect cell toxicity in our tests for ATIII. Our experiments yielded routinely SI values of greater than 10. We found that inhibition was HA dependent with decreasing efficacy in the order of H1N1 > H3N2 > H5N1 >> Flu B. Ribavirin yielded an EC50 between 12.3 μM (NR assay) and 5.3 μM (Visual Assay) against influenza A/California/07/2009 (H1N1) virus. Thus, ATIII was 96-fold (NR Assay) or 177-fold (Visual Assay) more active than ribavirin for this strain. ATIII elicited inhibition for influenza A/Perth/16/2009 (H3N1) that was 390-fold (NR Assay) and 238-fold (Visual Assay) stronger than ribavirin. There was a 390-fold (NR Assay) or 150-fold (Visual Assay) greater ATIII treatment effect than ribavirin against Influenza A/Duck/MN/1525/81 (H5N1).

In vitro efficacy of ATIII in human influenza virus strains measured as EC50 and EC90 by neutral red, visual and virus yield assay and measurement of toxicity by IC50.

Concentrations are in nM.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Assay</th>
<th>EC50</th>
<th>EC90</th>
<th>IC50</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flu A (H1N1)</td>
<td>California/07/2009</td>
<td>Neutral Red</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;23</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>New Caledonia/20/99</td>
<td>Neutral Red</td>
<td>&gt;1200</td>
<td>&gt;1200</td>
<td>&gt;31</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>NW3/33</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;27</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>Solomon Islands/03/2004</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;13</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>PR/8/34</td>
<td>Neutral Red</td>
<td>&gt;1340</td>
<td>&gt;1340</td>
<td>&gt;1.3</td>
<td></td>
</tr>
<tr>
<td>Flu A (H2N2)</td>
<td>California/7/04</td>
<td>Neutral Red</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;7.1</td>
<td></td>
</tr>
<tr>
<td>Flu A (H2N2)</td>
<td>Victoria/3/55</td>
<td>Neutral Red</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;2.2</td>
<td></td>
</tr>
<tr>
<td>Flu A (H2N2)</td>
<td>Perth/16/2009</td>
<td>Neutral Red</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;1.3</td>
<td></td>
</tr>
<tr>
<td>Flu A (H5N1)</td>
<td>Vietnam/1932/2004H1</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;1.3</td>
<td></td>
</tr>
<tr>
<td>Flu A (H5N1)</td>
<td>Duck MN/1525/31</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;33</td>
<td></td>
</tr>
<tr>
<td>Flu B</td>
<td>H5/2006b</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;1.9</td>
<td></td>
</tr>
<tr>
<td>Flu B</td>
<td>Shangai/361/02</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;1.3</td>
<td></td>
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<tr>
<td>Flu B</td>
<td>Shangai/370/99</td>
<td>Visual</td>
<td>&gt;1720</td>
<td>&gt;1720</td>
<td>&gt;1.2</td>
<td></td>
</tr>
</tbody>
</table>

In vitro mouse-adapted influenza virus inhibition by ATIII

We also measured ATIII inhibition using mouse-adapted influenza virus strains to select the most suited strain for our in vivo testing. Most strains were strongly inhibited by ATIII with
16-176-fold higher efficacies than that found for ribavirin in the control experiments (Tab. II).

TABLE II.
In vitro efficacy of ATIII in mouse-adapted influenza virus strains measured as EC50 by neutral red and visual assay compared to ribavirin. SI is given for ATIII. Concentrations are in nM.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Assay</th>
<th>EC50</th>
<th>SI</th>
<th>Fold Ribavirin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flu A (H1N1)</td>
<td>New Caledonia/20/09-m</td>
<td>Neutral Red</td>
<td>100&gt;50&gt;20 &lt;10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>NWS/33-m</td>
<td>Neutral Red</td>
<td>133&gt;13&gt;13 &lt;13</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>Solomon Islands/03/2006-m</td>
<td>Neutral Red</td>
<td>114&gt;14&gt;14 &lt;14</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>PR/8-34-m</td>
<td>Neutral Red</td>
<td>150&gt;12&gt;12 &lt;12</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>Duck/MN/15/25/81-m</td>
<td>Neutral Red</td>
<td>258&gt;7&gt;7 &lt;7</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Flu A (H1N1)</td>
<td>PR/8-34-m</td>
<td>Visual</td>
<td>&gt;1720&gt;1720&gt;1720 &lt;1720</td>
<td>0 &lt;13</td>
<td></td>
</tr>
</tbody>
</table>

In vivo efficacy test using mouse-adapted influenza virus
We then tested the effects of treatment with ATIII or ribavirin using an influenza A/NWS/33-m (H1N1) virus infection in mice. ATIII III (400 µg (6.9 nmol), 200 µg (3.45 nmol), 100 µg (1.72 nmol)/mouse) in 50 µl was administered intranasally once a day for 5 days starting 2 hours prior to virus challenge. Seventy-five mg/kg (300 µM/kg) ribavirin was tested as a control with twice daily intraperitoneal (i.p.) administrations. Influenza A/NWS/33-m (H1N1) virus was administered intranasally at approximately 5 x 10⁷ pfu (~3 x mL50).

Using 10 mice per group, we observed a 20% reduction in mortality in the high-dose group (344 nM/kg or 20 mg/kg) when mice were evaluated over a 21-day period compared to a 100 % reduction in the ribavirin control group (Fig. 1A). Treatment with ATIII delayed the mean time to death, but the results were not statistically significant compared to placebo. We found that the two surviving mice recovered faster in body weight compared to the ribavirin group (Fig. 1B).

IV. DISCUSSION
ATIII showed superior in vitro efficacy compared to ribavirin, which was not confirmed in vivo. Our in vivo data suggest that we might need to change the intranasal administration to inhalation administration to increase the possible drug load in the lung tissue. Additionally, it might be necessary to change the formulation to increase mucin penetration. Nevertheless, we feel that ATIII is uniquely positioned to add to current treatment options. We have not tested at which step ATIII blocks viral replication. This was beyond the scope of this publication. It is possible that ATIII inhibits several steps in the virus’ lifecycle. Serpins are found to block entry and intracellular replication of HIV [18]. Additionally, the drug might neutralize lung proteases known to increase influenza virus replication, decrease lung tissue damage and lymphocyte infiltration as described earlier for another serine protease inhibitor [10]. ATIII might also have further advantages: ATIII limits bacterial outgrowth and lung injury in Streptococcus pneumoniae pneumonia when administered via inhalation suggesting a novel role as a cationic antimicrobial protein [19]. This might be useful during influenza virus infections with Streptococcus pneumoniae coinfections. These coinfections are typically associated with greater loss of lung tissue remodeling/wound repair, higher inflammation, more Ca²⁺ signaling and apoptosis than influenza infections alone, and are suspected to be the reason for the severity of the 1918 influenza virus and 2009 H1N1 pandemic [20].

There is a lack of activation of repair processes after influenza virus infection that might be restored by ATIII since there are disease scenarios where ATIII restored lung’s repair mechanism. This was true for example after smoke inhalation-induced lung injury [21].

A vast knowledge base of coagulation related treatment in the lung exists [22-25] which lowers the risk of the drug’s development. Additionally, ATIII is very stable against degradation: it has a 72 h half-life in blood much longer than most small molecule inhibitors.

In summary, our data indicate that ATIII might block HA activation by a host protease(s) necessary to gain its fusion ability to host cell membrane and thereby block the infection
process. It is also conceivable that the use of ATIII may exert additional indirect beneficial effects by suppressing proteases that are released from infiltrating immune cells directly or by desensitizing of inflammatory cell receptors. This anti-inflammatory activity may suppress a hyper-inflammatory response in severely influenza virus infected individuals, which has been described to be detrimental in humans and in animals.

V. CONCLUSIONS

Our data describe the inhibition of influenza virus H1N1 through serpin ATIII. We also found inhibition of other influenza strains with decreasing efficacies H1N1>H3N2>H5N1>>Flu B. ATIII was routinely more effective than ribavirin for all strains tested except Flu B. The in vitro efficacy did not transfer to feasible in vivo inhibition. Lack of in vivo efficacy calls for the use of new formulation techniques to target lung tissue and allow sufficient penetration. This was also true for another anti-viral indication of ATIII where only high in vivo efficiencies (up to 50,000-fold of current treatments) were generated after applying of a tissue and cell targeting ATIII-formulation [13].

VI. CONFLICT OF INTEREST STATEMENT

We have no conflict of interest to declare.

References


