DEVELOPMENT OF A NEW ANTIVIRAL MEDICATION FOR THE GRIPPE

Valentina Divocha
Ukrainian Research Institute for Medicine of Transport, 65039, Kanatnaya 92, Odessa, Ukraine

The grippe till now is a mass disease resulted in hospitalization of thousands of sick persons and high mortality rate. The objective of the work presented is to extract inhibitor of trypsin-like proteinases from the waste the first stage of the industrial process of gamma globulin preparation from donated human blood, and to study its protective properties at the flu under experiment. Derivation the pure inhibitor included the following stages: the enzyme excretion, ultrasonic disintegration of cells, ion-change chromatography on DEAE-cellulose, dialysis, lyophilic drying. The method described allows obtaining five isoforms with inhibitory activity. The greatest amount of trypsin-like proteinases was registered in the isoform of the 5th fraction. The 5th isoform with high inhibitory activity was used for the study of therapeutically properties and the experimental grippe A in white mice. The isoform mentioned showed a promoted protective effect.

Keywords: Inhibitors, proteinases, virus grippe, chromatography

Email: divocha09[ats]ukr(dot)net

Corresponding contact: Ukrainian Research Institute for Medicine of Transport, 65039, Kanatnaya 92, Odessa, Ukraine

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Introduction

Flu still remains one of the most wide-scale diseases that result in hospitalization of thousands of patients and high mortality. 10-14 million people got sick from the flu every year in Ukraine and it accounts for 25-30% of the total morbidity. The morbidity rate remains high and causes a lot of damage. At present, the mortality from these diseases and its complications is not reduced, but on the contrary, there is this index stabilization and increase (Mitamura and Sugaya, 2006). In 2010 the flu caused 1127 fatalities in Ukraine, among them there were 100 pregnant women in the 2nd and 3rd trimesters.

Therefore, the search of new drugs for the prevention and treatment of the influenza and acute virus respiratory diseases (AVRD) is still urgent; it is topical issue particularly in the early stages of the disease, taking into account the limited capacity of practitioners in the differential diagnosis of these diseases at the pre-hospital stage. To obtain a drug for the influenza, we used "proteolytic" hypothesis of the influenza virus development in humans (Veremeyenko, 1994). Proteolysis has a special role in the process of inflammation, which initiates a cascade mechanism of universal non-specific activation of proteolytic enzymes on the local and systemic levels. Evolution has created mechanisms for the regulation of proteolysis as its inhibitors, which is available even in microorganisms (Veremeyenko, 1988).

According to our data the wastes of sera production are a promising initial biomaterial for trypsin-like protease inhibitor obtain with antiviral properties and lower allergenicity.

The objective of the work presented is to extract inhibitor of trypsin-like proteinases from the waste the first stage of the industrial process of gamma globulin preparation.
from donated human blood, and to study its protective properties at the flu under experiment.

**Materials and methods**

Strains of influenza virus: A/PR/8/34 (H1N1), grown on 9-day chicken embryos, were obtained at the D.I. Ivanovsky Research Institute of Virology, Academy of Medical Sciences of Russia. The influenza virus A/PR/8/34, white mice weighed 16-18 g, industrial wastes of fractions (II + III) gamma globulin and albumin manufacturing from donated human blood.

### 2.1 Virological analysis

The procedure included infection and accumulation of influenza virus A/PR/8/34(H1N1) on chicken’s embryos; adaptation of influenza virus A/PR/8/34 to white mice. Four passages of influenza virus have been done. A fatal dose of influenza virus equals to 5 LD₅₀ has been obtained. The choice of the dose of trypsin-like proteinase inhibitor for the treatment of white mice infected with IAV.

### 2.2 Biochemical analysis

We used chromatographic methods for extraction and purification of inhibitor from the wastes of sera-manufacturing industry. Protein determinations were done in O. Lowry method, determination of trypsin inhibitor activity by casein's hydrolyze in K.M. Veremeyenko method, modified by A.P. Levitsky. Electrophoretic analysis was done in U.K. Laemmli method.

### 2.3 Extraction and purification of trypsin inhibitor from lungs of mice

Extraction of inhibitor was done by the method used for proteinases extraction. Trypsin inhibitor purification was done by ion-exchange chromatography on DEAE-cellulose, gel-filtration - on Sephadexes G-15 and G-50, by affine chromatography on trypsin - sepharose 4B. In the last-mentioned case trypsin was covalently perfected in 0.05 M tris-HCl buffer pH 7.6. Desorption was done consequently with buffer solutions, containing 1.0 M NaCl, 8.0 M urea and 0.2 M KC1-HCl pH 2.0 solution. Trypsin’s inhibitor in fractions was determined by deceleration of benzoyl-arginine-p-nitroanilide hydrolysis by crystalline trypsin (Levitsky, 1979).

### 2.4 Method of determination of inhibitor activity

Determination of proteinases inhibitors in lungs homogenates, blood serum, and allantois liquid was done by casein’s method offered by A. P. Levitsky. To put 0.2 ml of supernatant into new glass tubes. To add 2.0 ml of reagent A and 2 ml of Folin’s reagent. Contact - 30 min at room temperature. Analyze at spectrophotometer.

**Calculate of inhibiting activity (IA)**

Serum:

\[
IA = \frac{(\Delta E_{tr} - \Delta E_{0n}) \times 0.2 \times n}{\Delta E} \quad \text{g/l; mg/ml}
\]
Tissue:

\[ IA = \frac{(\Delta E_{tr} - \Delta E_{0n}) \times 200 \times 21 \times m}{\Delta E_{tr}} \]

where: $\Delta E_{tr}$ - extinction of the sample with trypsin; n - dilution of the solution with serum; 0.2 - trypsin’s concentration, mg/ml; m - dilution of inhibitor’s solution; 200 - the amount of trypsin in 1 ml (200 mkg); 21 - ratio of tissue’s charge to extragent, weight 100 mg. per 2 ml; 1000 - recalculation coefficient against 1 gr of tissue; I - content of inhibitor per 100 ml; 1 - a unit corresponding to 1 gr of crystalline trypsin; E0n - extinction of the sample with the mixture trypsin + inhibitor.

2.5 Statistical analysis

The results of the investigations carried out have been processed with the programmer "Microsoft®Excel".

Results

At the first stage of industrial gamma globulin production (Cohn’s method), centrifuge is utilized. However, according to our data, this centrifuged contained 481.11 g of trypsin - like proteinases inhibitor per 1.0 kg of raw material (Table. 1).

<table>
<thead>
<tr>
<th>N</th>
<th>Sample</th>
<th>Protein, mg/ml</th>
<th>Inhibitor, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supernatant</td>
<td>19.36 ± 1.64</td>
<td>0.481 ± 0.046</td>
</tr>
<tr>
<td>2</td>
<td>Precipitate</td>
<td>11.07 ± 1.01</td>
<td>0.058 ± 0.004</td>
</tr>
</tbody>
</table>

Inhibitor content in the supernatant was 8.0 times higher than its content in the sludge. Therefore, we used the supernatant for the isolation and purification of the inhibitor of trypsin - like proteinases by ion exchange chromatography. The method of producing the inhibitor in purified form comprised the following steps: extraction of the enzyme, ultrasonic disintegration, ion exchange chromatography on DEAE-cellulose 52, dialysis, freeze-drying (Ukrainian Patent N 89778) (Divocha, 2010). Ion exchange chromatography was performed in 0.1 M phosphate buffer, pH 7.5. Linear gradient was created in the range of 0.0-1.0 M NaCl in the same buffer. This method allowed obtaining five isoforms possessing inhibitory activity (Figure 1). The first two isoforms, containing trypsin-like proteinase inhibitor was eluted from ion exchange column with 0.1 M phosphate buffer pH 7.5. The following three isoforms, containing inhibitor of trypsin - like were eluted by stepwise gradient of NaCl with different molarity: the 3rd isoform - 0.1 M NaCl, the 4th - 0.2 M NaCl, the 5th - 0.5 M NaCl. Volumes of the isoforms eluates were respectively: 35 ml, 195 ml, 340 ml, 440 ml, 605 ml. The highest content of trypsin-like protease inhibitor was recorded in the fraction of the 5th isoform, which was the latter eluted from
the column 0.5 M NaCl, and the least - in the 4th and 3rd isoforms which eluted from the column with 0.2 M and 0.1 NaCl, correspondingly. These results obtained indicate that in industrial wastes of the I\textsuperscript{st} stage of gamma globulin production 5 fractions of the inhibitor of trypsin-like proteinases, which differed from each other both by the charge as well as for salt-soluble.

**FIGURE 1. ISOLATION AND PURIFICATION OF THE TRYPSIN-LIKE PROTEINASES INHIBITOR FROM THE WASTES OF THE I\textsuperscript{ST} STAGE (II + III) OF \textgamma-GLOBULIN INDUSTRIAL MANUFACTURING**

These differences may be due to differences in amino acid composition of multiple forms of the inhibitor under study.

The 5\textsuperscript{th} isoform with high inhibitory activity (132 units) and trypsin-like proteinases low activity (0.0027 mmol/min in the sample) was used to examine the therapeutic properties at experimental influenza in the group of albino mice.

The mice were divided into 7 groups, 4 of them were experimental ones and included 15 pieces, control groups had 10 pieces (Table. 2). The first group animals got a lethal dose of virus (virus control). The virus was administered intranasal in a volume of 0.05 ml at Rausch-narcosis. The second group of mice received the same dose of the virus, but simultaneously they were exposed by the action of crystalline trypsin (control of the therapeutic properties of crystalline trypsin) at the same doses and timing as the 3\textsuperscript{rd} group animals did.

The 3\textsuperscript{rd} group of animals was infected with the same dose of the virus and was treated with trypsin-like proteinases inhibitor derived from the wastes of gamma globulin production. The 4\textsuperscript{th} group got only the inhibitor of trypsin-like proteinases obtained from the wastes (inhibitor toxicity control). The animals of the 5\textsuperscript{th} group were administered only crystalline trypsin (trypsin action control), the 6\textsuperscript{th} group got phosphate buffer with the virus diluted, inhibitor and trypsin (control of reagents). The 7\textsuperscript{th} group was a control of intact animals (they did not get any drugs).

Both inhibitor and trypsin were administered intranasal to each mouse under light ether anesthesia for seven days. Each mouse received 140 mg of the inhibitor for the course of treatment.

The study of outcomes showed that the 1\textsuperscript{st} and 2\textsuperscript{nd} groups animals died in 3-8 days after infection. The significant difference in the terms of death and the clinical picture of these
groups of animals was observed, for example the 2nd group mice started to die much earlier (in 3 days after infection).

***It can be concluded that the additional administration of trypsin accelerated the flu course after administration of the virus lethal dose, since the death of mice occurred somewhat earlier. In the third group 12 white mice survived (80%), they remained alive and on the 14th day after infection (total time of observation). Consequently, the inhibitor blocking trypsin-like proteinase largely suppressed the infection process, induced by influenza virus.

The 4th, 5th, 6th and 7th groups of animals remained alive throughout the period of observation. In addition, newly obtained inhibitor of trypsin-like proteinases did not cause toxicity as the 4th group of white mice was still alive on the 14th day after administration of inhibitor.

**Table 2. Action of the Fifth Isoform of Trypsin-like Proteases Inhibitor on the Survival of Mice Infected with a Lethal Dose (2.5-2 LD50) Influenza Virus A/PR/8/34**

<table>
<thead>
<tr>
<th>N</th>
<th>Group name</th>
<th>Number of animals</th>
<th>Dose of virus</th>
<th>Dose of inhibitor per mouse, mkg of protein</th>
<th>Number of animals</th>
<th>% of the animals survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Influenza Virus A</td>
<td>15</td>
<td>10^-1</td>
<td>-</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Influenza Virus A + trypsin crystal</td>
<td>15</td>
<td>10^-1</td>
<td>20 mkg</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Influenza Virus A + inhibitor from wastes blood</td>
<td>15</td>
<td>10^-1</td>
<td>20 mkg</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Inhibitor from wastes (toxicity)</td>
<td>15</td>
<td>-</td>
<td>20 mkg</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Trypsin crystal (control)</td>
<td>10</td>
<td>-</td>
<td>20 mkg</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Phosphate buffer (control)</td>
<td>10</td>
<td>-</td>
<td>0.2 ml</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Control of animals</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Thus, the results of the study suggest that the preparation of trypsin-like proteinases inhibitor obtained from the wastes of γ-globulin 1st stage manufacturing had distinct antiviral properties. The results obtained are convincing experimental substantiation for inhibitor of trypsin-like proteinases use for the treatment of viral influenza. Inhibitor can be used not only for influenza treatment, but for the treatment of other viral infections, if the cleavage of protein - precursor of viruses is performed by cellular trypsin-like proteinases. The latter include a large group of diseases - from viral hepatitis to AIDS.

**Discussion**

Problems of influenza therapy are still urgent, because the methods of its prevention and specific immunotherapy are not effective enough because of marked variability of influenza viruses (Hendon, 2009). In view of the said above, the value of preventive vaccines, although is very high, but they do not provide complete protection of the population from influenza, even in the case of timely application of highly specific vaccines.

Influenza vaccines do not exclude the possibility of allergic reactions to serum proteins or proteins of chicken embryos that significantly limits their application. This is especially real at the manufacture of live influenza vaccine, which almost does not pass a special purification from proteins of chicken embryos (Hendon, Markushin, Anopova et al., 2005).
Search of the material from which the drug with high antiviral activity (inhibitor of trypsin-like proteinase) can be obtained, and at the same time having the lowest allergenicity to humans, drew our attention and made us conduct studies of human blood, and the wastes of serum production for the presence there of serine proteinases and their inhibitors.

From the literature it is known that blood serum is an important source of various inhibitor of proteinases. To the inhibitors currently known include: α2-antiplasmin, α2-macroglobulin, α1-antitrypsin, antithrombin III, C1-activator, α1-antichymotrypsin, inter-α-inhibitor of trypsin, et al (Veremeyenko, 1988). Although, we suppose that this list does not exhaust the number and variety of proteinase inhibitors of human blood plasma. From the damaged cells of various organs the components of proteinase/inhibitor systems of intracellular origin may come in blood.

Taking into account that the production of the inhibitor from the tissue of murine lungs cannot have commercial value, we have modified it and developed a new method of producing inhibitor of trypsin-like proteinases from the wastes of serum industrial production from human donor blood and tested it as a therapeutic drug (Divocha, Mykhalchuk, and Gozhenko, 2006a).

According to the method proposed, for the isolation of trypsin-like proteinases inhibitor we used the wastes of the I<sup>st</sup> stage (II+III) of industrial production of gamma globulin from human blood that contained a significant amount of the inhibitor mentioned (Divocha, Mykhalchuk, and Gozhenko, 2006b; Divocha and Mikelashvily, 2001). This method yielded to obtain five isoforms with inhibitory activity. All the inhibitors obtained were studied by their activity. The highest inhibitory activity of trypsin-like proteinases was registered at the 5<sup>th</sup> isoform fraction. We have improved the method of allocation of trypsin-like proteinases inhibitor and got a patent of Ukraine № 21599 from 15.03.2007 (Divocha, Mykhalchuk, Gozhenko, 2006a).

We examined the protective effect of the V<sup>th</sup> isoform of trypsin-like proteinases inhibitor got from industrial wastes of the I<sup>st</sup> stage. According to our research, the V<sup>th</sup> isoform trypsin-like proteinases inhibitor protected animals infected with a lethal dose of influenza virus for 80%.

Administration of the V<sup>th</sup> isoform of trypsin-like proteinases inhibitor, its protective effect could be manifested not only through their blockade and inhibition of infection, but also due to the parallel inhibition of tissue proteinases involved in the reactions of immune inflammation, which contributes to viral lung damage.

Preparation of antiviral drugs from the wastes of donor blood allows using better human blood proteins, enhance the economic viability of the fractionation, increase the list of blood products, and low the cost of their manufacture.

In general, inhibitory therapy of viral influenza should be considered as a promising new direction in the treatment of this disease and its complications.

Due to the fact that this direction of treatment is based on a common for many viruses mechanism of deproteination, it should be assumed that the treatment of many viral diseases can also be based on the modulation of trypsin-like serine protease/inhibitor. Among them are viral hepatitis, AIDS, and many others.

Along with the main process of proteinases suppression by inhibitors administration stimulation of proteinases inhibitors synthesis and their activation may be promising.

This pathogenic method of viral diseases therapy should be fully analyzed as one of the promising areas of general biology, regulating the relationship between viruses and human’s body.
References


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