Juniper Online
Journal of Immuno Virology
ISSN: 2473-5582

Juniper PUBLISHERS
key to the Researchers

Research Article
Volume 2 Issue 3 – July 2017
DOI: 10.19080/JOJIV.2017.02.555586

JOJ Immuno Virology
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Role of a Body's Proteinase-Inhibitory System at Grippe

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Submission: June 16, 2017; **Published:** July 24, 2017

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Summary

A new theory of grippe pathogenesis with participation of proteinase-inhibitory system has been offered. It has been established that purification and concentration of grippe viruses by different methods did not release the virus from cellular enzymes. At the experimental animals infecting with the virus of grippe, disturbance of enzyme-inhibitory balance took place, especially during first hours after the animals being infected. From the lungs of healthy mice, they have got six isoforms of trypsin-like proteinases. To all of them they got antiproteinase immune sera and have treated the experimental animals. It was antiserum to the third isoform that has prevented the experimental animals fatality.

Introduction

In the pathogenesis of viral diseases the interaction of virus and cell is under-investigated. Penetration of a virus into a healthy cell with virus's obligatory deproteinization is of top importance here. While viruses deproteinization is studied insufficiently. First of all it refers the mechanisms of grippe virus's penetration into the cell of mammals, including a human being. In this respect win 1983 we offered a new theory of grippe pathogenesis with proteinase-inhibitory system (PIS) participation [1,2]. The difficulties of new antiviral drugs creation rise from specific biological properties of viral diseases pathogens. The recent findings of biochemistry and molecular biology have highlighted some peculiarities of virus' reproduction and provided new approaches for a directed interference into the cycle of viral reproduction [3,4].

The objective

To examine the state and role of antiproteinase systems of virus and recipient in the development of influenza infection and obtained fundamentally new medicinal preparations based on of trypsin-like proteinases inhibitors.

The tasks

- To learn the proteinases' and their inhibitors role at different, especially early stages, of grippe development.
- To eliminate and purify proteinase and its inhibitor from the lungs of healthy and infected by grippe mice.
- To investigate the protective properties of cellular inhibitor at infection of the animals with lethal dose of grippe virus.

Results

At early 1980 when purified and concentrated different races of grippe viruses to obtain polyvalent anti- grippe vaccines we met with impossibility to release influenza's virus from protolithic activity. To solve this problem we have improved the methods of purification but nevertheless we failed to purify influenza virus from protolithic activity [5].

Analysis of purified preparations of influenza virus showed that ultracentrifugation does not release influenza's virus from protolithic activity and in saccharose's gradient (15-60%) it distinctly got into several isoforms (Table 1).

The results obtained allowed us to conclude that serine-containing proteinase of trypsin-like type of cellular origin with molecular heterogeneity is associated with influenza virus [6,7].

System of proteinases and inhibitors is presented in the body by a vast group of proteins. Inhibitors of protolithic enzymes have a role of the constant level of corresponding enzymes and are in a constant dynamic equilibrium with the latter. Disturbance between enzymes and inhibitors is of importance for the development of pathological processes.

Table 1: Protamine-shifting and hemagglutinating activity of influenza virus in fractions of sugar gradient to neutralization and after neutralization by immune (CAM) rat sera.

Expirements	No of Sugar Gradient fraction	% of Sucrose	Proteinase before Neutralization, mkg/arg per	Proteinase After	Ha Before Neutralization in	Ha After
			Min in 1ml	neutralization, mkg/arg per	0.1ml	neutralization
				Min in 1ml		In 0.1ml
1. Influenza Virus V Virus	1	5	1.42	0	0	1:02
	2	15	32	0	1:08	1:02
	343	32	8.5	0	1:16	1:08
	4	42	29.8	0	1.463888889	1.4638889
	5	49	3.9	0	0.769444444	0.3972222
	6	52	32.9	0	0.086111111	0.0861111
	7	55	32.2	0	0.086111111	0.0861111
	8	57	6.04	0	1:02	1:02
2. Influenza Virus	1	3	1.3	0	0	0
	2	11	12		1:02	1:02
	3	24	7.28	0	1:16	1:16
	4	24	9.42	0	1:16	1:16
	5	38	84.6	0	0.397222222	1.4638889
	6	42	23.28	0	1.463888889	0.7527778
	7	46	10.8	0	0.752777778	0.3972222
3.CAM	1	6	0.05	0	0	0
	2	17	1.101	0	0	0
	3	27	0.037	0	0	0
	4	29	0.059	0	0	0
	5	37	0.08	0	0	0
	6	41	0.143	0	0	0
	7	49	0.064	0	0	0
	8	53	0.128	0	0	0
	9	56	0.108	0	0	0

CAM: Chorionall antoic membrane; HA: Hemagglutinating activity.

Our researches show that the level of proteinase activity and activity which inhibits proteinase is in equilibrium in the lungs and blood serum of uninfected animals and the latter disturbances at infection by the virus of influenza A [8].

At communicable process the deepest changes have place during first hours after introduction of infection. Thus, in 6 hours after contamination the amount of proteinase in lungs and blood serum of the infected animals reduces while inhibiting activity increases. The cells contaminated by influenza virus induce appearance of inhibitor both in lung tissue and in blood serum. So, lung inhibitors are an organ "first line of resistance"at the action of various flu strains [9].

6 isoforms of trypsin-like proteinase were extracted from the lungs of the healthy mice and 8 isoforms from the infected animals by ion-exchange chromatography. Their specific proteolytic activity vastly increased compared to the source material. The proteinase isoforms' obtained had a wide range of substrate specificity and were able to hydrolyse substrates of both natural and synthetic origin (Table 2).

Table 2: Purification DEAE –cellulose-53 of lung trypsin-like proteinase of healthy mice.

N of Fraction	N of Isoform	Specific Proteolic Activity per mg of Protein	% of Proteinase Outcome	% of Purification by Protein
33	I	4.285	2.09	96.8
53	II	83.75	5.84	99.07
65	III	22.42	2.703	98.38
75	IV	40.00	6.279	97.92
121-130	V	32.6	136.74	99.98
161-189	VI	0.787	421.74	64.90

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Antiproteinase hyper immune rat sera were obtained to both isoforms of trypsin-like proteinases [10]. In studies of the protective properties of antiproteinase sera and serum of healthy rats on the white mice infected intranasal with the lethal dose of influenza virus A/PR/8/34 (IV passage) it was found that 100% of control mice fatality took place in 4-5 days. The animals to which they injected healthy rats serum intranasal 6 times, died on the 7th day. In the treatment of mice with the pools of immune sera I, II, IV, V and VI groups the animals mortality rate was reduced and lethality came much later than in the control group. 20% of the animals recovered (Table 3).

Table 3: Influence of antiproteinase immune sera on survival of mice under infection with lethal dose of influenza virusA/H1N1/PR/8/34.

N of gr. of mice	Isoform of Proteinase	Sera group	Terms After Infecting, Hours and Days												
			6h	1	2	3	4	5	6	7	8	9	10	14	Survived	% of survive
1.	I	I	0/10	0/10	0/10	010	0/10	2/8	0/8	2/6	2/4	2/2	0/2	0/2	2	20
2.	I	II	2/8	2/8	0/6	4/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2	20
3.	II	III	0/10	0/10	2/8	0/8	0/8	2/6	0/6	2/4	0/4	0/4	2/2	0/2	2	20
4.	III	IV	0/10	0/10	0/10	0/10	0/10	2/8	0/8	0/8	2/6	0/6	0/6	0/6	6	60
5.	IV	V	0/10	0/10	2/8	2/8	2/6	2/4	2/2	0/2	0/2	0/2	0/2	0/2	2	20
6.	V	VI	0/10	0/10	5/5	3/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2	20
7.	VI	VII	0/10	0/10	0/10	7/3	1/2	1/1	0/1	0/1	0/1	0/1	0/1	0/1	1	10
8.	Sodium chloride solution		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	10	100
9.	Serum-free virus		0/10	0/10	0/10	0/10	2/8	6/2	2/0	0/0	0/0	0/0	0/0	0/0	0	0
10.	Healthy rats serum		0/10	0/10	0/10	0/10	2/8	3/5	0/5	5/10	5/0	0/0	0/0	0/0	0	0
11.	Immune serum IV gr., virus-free (toxicity)		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	10	100

Note: 1. Numerator: number of mice died; 2. Denominator: number of mice in the experiment

The most effective was the fourth pool of immune serum to the IIIrd isoform. In its presence 60% of infected mice survived and on the 14th post-infection day in the blood serum and in the lungs we did not detect either hemagglutinin, no infectious virus. Immune serum to isoform VI did not protect mice from death at all, although isoform III differed from isoform VI by only one protein with molecular mass 32 kDa.

From the lungs of healthy mice, we have isolated an inhibitor of trypsin-like proteinases with molecular mass 47.5 kDa, with a high degree of purity and small amount of impurities. We have designed and patented a technique of procedure and decontamination of trypsin-like proteinases [11,12]. The inhibitor isolated is alike α_1- inhibitor of human blood serum (m.m. 48-55 kDa) and egg-yolk trypsin (m.m. 49 kDa) but is unlike the inhibitor isolated from the lungs of cattle (inhibitor of Kunitz-Northrop type) with molecular mass 65kDa. In studies of its effect on trypsin-like proteinases isoforms by tube test it has been revealed that it suppresses activity of practically all isoforms, excluding the 4th (41.8%) and the 8th (28.3%).

In our researches it has been revealed that the cellular inhibitor suppressed the development of grippe virus in the chicken's embryos and development of infectious and hemagglutinating activity and formation of the total protein. At the same time inhibitor of trypsin-like proteinases extracted from the lungs of previously viremic mice had no such a property. At our further researches for the treatment of grippe infection in animals we used inhibitor extracted from the lungs of healthy animals [13]. Administration of this inhibitor to the previously viremic by lethal dose of grippe mice reduced the percentage of their deaths because of HA decomposition inhibiting at virus's reproduction in lungs, arresting of the process generalization and due to the prevention of increase of proteolysis in lungs [14], precaution of aero-hematic barrier and some hyper reaction of local protection (Table 4).

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Table 4: Action of cellular inhibitor of trypsin-like proteinase of the survival rate of mice infected with lethal dose of influenza virus A/PR/8/34.

N and Name of Group of Animals (White Mice)	Number of Animals in the Group	Dose of Influenza Virus 5 LD_{50}	Dose of Inhibitor, µg on a Mice, One Injection	Number of Animals		% of Animals Protected from Virus
				Dies	Survived	
1.Influenza virus	40	10^{-3}	-	40	-	0
2. Influenza virus +trypsin, crystal.	40	10^{-3}	18µg	40	-	0
3.Influenza virus + inhibitor from healthy lungs	40	10^{-3}	18µg	7	33	82.5
4.Cellular inhibitor	40	10^{-3}	18µg	-	40	100
5.Trypsin, crystal	10	-	18µg	-	10	100
6. Phosphate buffer	10	-	0.2ml	-	10	100

Discussion

One of the most important stages of the development of many viruses in the host organism is their introduction in the cell after preliminary deproteinization. Regulation of this development of virus is one fundamental principles of their reproduction. Induction or intake of inhibitor of virus proteolytic activation is one of the promising ways of viral diseases treatment, including influenza.

Methods

Strains of influenza virus: A/PR/8/34(H1N1), grown on a 9-day chicken embryos, were obtained at the D.I. Ivanovsky Research Institute of Virology, Academy of Medical Sciences of Russia and strain AO/32 (H1N1) - from the Influenza Research Institute of St. Petersburg, Russia; white mice and hybrids; chicken embryos; white rats, Wistar line.

Virological analysis

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 has 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.

Concentration and purification of influenza virus

Influenza virus AO/32 (H1N1) with infectious titers 7.0 log $EID_{50/0,2}$ml and hemagglutinin (HA) -1:256. To obtain influenza virus preparations we used 10-11-day chicken embryos. The virus was accumulated by infecting chicken embryos in a volume of 0.2ml, diluted to 10^{-3} with infectious material. Infected chicken embryos were incubated for 48 hours at +36 °C. Then they were cooled for 18 hours at +4 °C and then the virus-containing fluid was collected, purified and concentrated with centrifugation.

Concentration and purification of influenza virus was done in as follows: virus-containing liquid was consecutively centrifuged with 6000 RPM during one hour at + 4 °C on centrifuge CLR-1 (Ukraine) for purification from components of tissue and erythrocytes. Then 30min at 10000 R.PM at +4 °C on centrifuge CLR-1 (Ukraine) to remove the main mass of cellular proteins. After that virus was precipitated at 2000 RPM during an hour on centrifuge CLR-1 (Ukraine) through 20% layer of sucrose for more complete purification of virus. Purified and concentrated virus was additionally purified with speed centrifugation in sucrose density gradient (15-60%) on centrifuge VAK-602 in backet-rotar at 28000 RPM during four hours. Influenza virus which was in the zone 37-45% of sucrose was re-ultra centifugated at 33000 RPM in rotor SV-40, ultracentrifuge Spinko, three hours.

In the preparations obtained and eluates of fractions the content of proteins in (Lowry, 1951) method was controlled after dialysis at all stages. As well as content of HA in routine method with 1% solution of chickens embryo erythrocytes and the presence of proteolitic activity in KNVeremeyenko method [16] in SV Vovchuck's [17] modification.

Study of trypsin-like proteinasis role in the development of viral infection in mice

In the present work we use influenza virus A/PR/8/34 (H1N1) adopted to lung tissue of white mice; virus' infectious titer was 7.0 log$EID_{50/0,2}$ml and HA titer 1:128. Outbred white mice with mass of 16-17gr were taken in the experiment. Contamination of the animals was done at a light ether intranasal anesthesia, volume 0.05ml at the dilution 10^{-3}, which corresponded influenza infectious dose $5LD_{50}$. This dose afforded 100% animals' fatality on the 6th day after contamination. The animals were killed and their lungs taken off (5 mice in each group) in 15 and 30min; 1 hour; 6 hours, etc. and later on 1-6 days after contamination. Concurrently, non-infected mice were

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killed and their lungs extracted (5 animals in each group) at the same terms. Blood was taken as well. Lungs were washed in cold 0.01M phosphate buffer pH 7.5 twice, grinded in a cold mortar, suspended in phosphate buffer (1ml for one lung), homogenized with ultrasound at the mode 7 on High Intensity Ultrasohie Procession, Chicago Cole Parmel (USA), centrifugated at 10000 RPM on the centrifuge RS-5 (Sorvall Instruments, Rotor SS-34), during 1 hour, temperature +4 °C. Supernatant and blood serum was used for determination infectious, proteinase and proteinase inhibiting activities. Virus infectious titer in the lungs of infected mice and allantois liquid was determined by contamination 9 -10 days chicken embryos and expressed it in log$EID_{50/0,2}$ml.

Determination of trypsin inhibitor activity by casein's hydrolysis was done in KM. Veremeyenko method, modified by AP Levitsky [18].

Learning of virus influence on proteinase-antiproteinase activity

Influence of influenza virus on the dynamics of proteinase and inhibiting activity has been investigated at multiplication of virus in chicken embryo. Virus A/H1N1/PR/8/34, adopted to chicken's embryo with HA titer 1:128 was used for contamination of 11-day chicken embryos in two doses: a large dose (at the dilution 10^{-1}, that corresponded to $2,5^{-2}LD_{50}$) and a small one (at the dilution 10^{-6}, that corresponded 1 LD_{50}).

Virus-containing allantois liquid was taken in 15 and 30min., 1 hour, 24, 48, 72, 96 and 122 hours after contamination. Three embryos were used for each period. Uninfected chicken embryos were used as a control. Allantois liquid was taken in them in 15 and 30min. 1 hour, 24, 48, 72 and 122 hours (three embryos for each term) and learnt the same indexes as in contaminated material. In the material selected we learnt the presence of proteinase, inhibiting and infectious activity, HA and protein.

Biochemical analysis

We used chromatographic methods for extraction and purification of inhibitor from the wastes of sera-manufacturing industry. Protein determinations was done in OH Lowry method, determination of trypsin inhibitor activity by casein's hydrolyse in KM Veremeyenko method, modified by AP Levitsky. Electrophoretic analysis was done in UK Laemmli method [19].

Extraction and purification of trypsin-like proteinases

Trypsin - like proteinases have been extracted from the lungs of the healthy mice (100 items for an experiment) and also from the lungs in 72 hours after contamination with influenza virus A/H1N1/PR/8/34(100 items for an experiment). The experiment was performed with the mice of the line «Balb/c», weighted 16-18gr. Contamination of the animals with the virus of influenza was performed under light ether anesthesia in the volume 0.05ml in the dilution 10^{-6}, which corresponds virus infectious dose 1 LD_{50}.

In 72 hours after the animals' contamination the animals were killed and their lungs were extracted. Parallels with it blood was taken. The lungs were shredded with sissors, then homogenized in a mortar, processed with ultrasound at 18 kHz, 75 "on the device Soniprep 150 MSE. The whole work was done at the temperature +4 °C. Homogenate was centrifuged at 10000 RPM during 1 hatcentrifuge RC, temperature +4 °C. Supernatant I was freezed, 1% Triton X-100 was added to the precipitate and left in the refrigerator till morning. Next day the precipitate was processed with ultrasound once more and centrifugated at the same regimes. Supernatant II was taken. Both supernatants (I and II) were joined later on for purification.

Purification of the material dialyzated was done by ion-exchange chromatography on DEAE-cellulose-32, deviceZKB - 2023, Minicololab (Broma). Colomn's height was 18cm, diameter 1.75cm. The extent of purification was controlled by proteins and protease's activity. Fractions with high indexes of protease were purified additionally by dialysis, then dried by liophilization onHetociteHeto (Denmark). Extracted and purified isoforms were used for immunization of the animals. At all stages of isoforms of trypsin-like proteinase purification, in all fractions determination of proteinase, inhibiting, hemagglutination activity and total protein was carried out, 19 biological repetitions were done.

Production of hyperimmuneantiproteinase sera

To obtain sera we used white rats of Wistar line weighed 170-200 gr. White rats were immunized four times, once per week with each isoform of trypsin-like proteinase extracted from the healthy and infected lungs of mice with a complete Friend's adjuvant. Each rat got 560 units of protein and 890 units of proteinase. Total pick up of hyperimmune sera was done in 7 days after the last immunization.

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 0.05M tris-HC1 buffer pH 7.6. Desorption was done consequently with buffer solutions, containing 1.0M NaCl, 8M urea and 0.2M KC1-HCl pH 2.0 solution. Trypsin's inhibitor infractions was determined by deceleration of benzoyl-arginine-p-nitroanilide hydrolysis by crystalline trypsin [18].

Determination of trypsin-like proteinases activity by protamine hydrolysis

Qualitative reaction to arginine, forming at the hydrolysis of protamine and histones, and which does not precipitate with 20% CCl_3COOH, forms the basis the method.

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Reagents

- % solution of protaminesulphate on 0.1M phosphate buffer pH 7.5.
- 20% solution of CCl_3COOH.
- 0.004% solution of oxychinoline. 100mg of oxychinoline was diluted in 50.0ml of spirit of wine. The working solution was prepared by 50 times soluted with aqua distillate initial solution just before determination.
- 10.0% NaOH.
- 10.0% solution of NaBrO 1.0gr Br (0.3ml) was brought up to 100ml with cooled to 0 ºC 5.0% solution NaOH.
- 40.0% solution of urea.

Procedure of determination

To the mixture, containing 0.2ml of protamine sulphate and 0.5ml 0.1M phosphate buffer pH 7.5, 1ml of enzymatic solution was added. The samples were incubated 150min on the water bath at +38 °C. The reaction was stopped by 0.9ml of 20.0% CCl_3COOH. The content of the tube was mixed and centrifuged 15min at 6000 RPM 10ml of supernatant was got over into ice-cooled tubes. 1.0ml of oxychinolone solution, 1.0ml of 10% NaOH and 0.2ml of NaBrO was added in succession. The tubes were agitated and in 15min 1.0ml of 40.0% urea was added. 1.0ml of cooled distillated water was added and in 5min the activity was checked at 508ml (blue-green light filter). To the contral sample 0.8ml of 20% CCl_3COOH was added to 0.8ml of protamine sulphate and phosphate buffer before. 0.5ml 0.1M of phosphate buffer pH 7.5; 0.1ml of the solution under study; 0.8ml CCl_3COOH (20%). 0.2ml 1% solution of protamine sulphate on phosphate buffer we added to the control sample.

Spectrophotometer was maintained by the control to reagents - instead of 1.0ml of supernatant liquid 1.0ml of 10.0% of CCl_3COOH was added in the sample. The further determination was similar to that in the experimental sample.

Enzymes activity was determined with the following formula:

Homogenate of lungs $A=\Delta E_{\mu g} \bullet 1,6 \bullet 5$

$0,1 \bullet T \bullet 174,2$

Bloodserum $A= \Delta E_{\mu g} \bullet 1,6 \bullet 100$

$0,1 \bullet T \bullet 174,2$

Total formula $A= \Delta E_{\mu g} \bullet n \bullet 2$,(modified by S.V.Vovchuk)

$0,1 \bullet T \bullet k$

Where, A - activity of enzyme in u/ml. A unit of activity is equal to an amount of enzyme causes formation of 1 micromole of arginine per 1min of incubation; ΔE - extinctions difference between experimental and control sample; n - dilution of enzymatic solution; 1.6 - the total volume of sample; 2 and 5 - recalculation for the whole mixer under intubation; 100 - calculation on 100ml of serum; 0.1 - volume of enzymatic solution; T - time of incubation, min; 174.2 - molecular mass of arginine; K - coefficient of conversion of extinction amount to micromole of arginine. For conversation of extinction to arginine's mc mol we plotted a calibrated curve against standard solution of arginine.

Method of determination of inhibitor activity

Determination of proteinases inhibitors in lungs homogenates, blood serum and all antoic liquid was done by casein's method offered by AP Levitsky [18].

To put 0.2ml of supernatant into new glass tubes.

To add 2ml of reagent A and 2ml of Folin's reagent. Contact - 30 min at room temperature. Analyze at spectrophotometer.

Calculate of inhibiting activity (IA)

Serum: $(\Delta E_{tr} - \Delta E_{0n}) \cdot 0,2 \cdot n$

$IA = \frac{\qquad\qquad\qquad\qquad}{\Delta E}$; g/l; mg/ml

Tissue: $(\Delta E_{tr} - \Delta E_{0n}) \cdot 200 \cdot 21 \cdot m$

$IA = \frac{\qquad\qquad\qquad\qquad}{\Delta E_{tr} \cdot 1000}$

Where: ΔE_{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 1ml (200mkg); 21 - ratio of tissue's charge to extragent, weight 100mg. per 2ml; 1000 - recalculation coefficient against 1gr of tissue; I - content of inhibitor per 100ml; 1 - a unit corresponding to 1gr of crystalline trypsin; E0n – extinction of the sample with the mixture trypsin + inhibitor.

Statistical analysis

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

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How to cite this article: Divocha V A. Role of a Body's Proteinase-Inhibitory System at Grippe. J Virol Curr Res. 2017; 2(3):555586. DOI: 10.19080/JOJIV.2017.02.555586.

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DOI: 10.19080/JOJIV.2017.02.555586

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How to cite this article: Divocha V A. Role of a Body's Proteinase-Inhibitory System at Grippe. J Virol Curr Res. 2017; 2(3):555586. DOI: 10.19080/JOJIV.2017.02.555586.