REPORT

Study of cytotoxic and antiviral effects of Fullerene - (tri-amino-caproic acid) hydrate and trisamine (1:1) in HEp-2 cell culture on respiratory-syncytial virus, *Long* strain

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SUMMARY

Report: 16 pages, 3 figures, 1 table

Key words: respiratory-syncytial virus, cytotoxicity, antiviral activity, Fullerene - (tri-amino-caproic acid) hydrate.

The study object is respiratory-syncytial virus (RSV), ***Long*** strain. The study subject is Fullerene - (tri-amino-caproic acid) hydrate and trisamine substance (1:1).

The study objective is assessment of cytotoxic and antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate in HEp-2 cell culture toward respiratory-syncytial virus.

Study methods - virological and cytological.

The study has demonstrated that Fullerene - (tri-amino-caproic acid) hydrate substance is not toxic for HEp-2 cells in concentrations up to 100 μg/ml and has antiviral activity against respiratory-syncytial virus. Antiviral activity of the substance increased with increasing the substance concentration and decreased with increasing the virus dose. Inhibiting concentration (IC50) of Fullerene - (tri-amino-caproic acid) hydrate at multiplicity of infection 0.1 and 0.02 MOI was 5.376±1.39 μg/ml and 3.238±2.02 μg/ml, respectively. The substance demonstrated antiviral activity when added 2 hours before or simultaneously with the virus infection; when added 2 hours after infection it did not have effect on RSV replication.

INTRODUCTION

Respiratory-syncytial virus (RSV) is a virus that causes infectious respiratory diseases. This infection is most dangerous for young children; 70% of infants have respiratory-syncytial infection (RSVI) during the first year and almost every child is infected during the first three years of life. The level of mortality from the virus-caused complications in babies from 6 weeks to 6 months of age is one of the highest among infectious diseases. A complicated course of infection is also typical for aged people with cardiopulmonary diseases, immunodeficiencies, and people who underwent transplantation of organs. RSV is spread everywhere; outbreaks of RSV infections commonly begin in the fall and continue in winter often running together with influenza epidemics. The immunity formed as a result of RSVI is not long-lasting and does not provide complete antiviral protection; this causes lifelong recurrent infections (1). Parent RSV-specific antibodies present in babies' blood do not guarantee protection from the infection either. Mechanisms of antiviral protection are different at primary and secondary infections; this makes inefficient the use of inactivated vaccines. Moreover, the use of such vaccines leads to more severe forms of the disease in vaccinated compared to non-vaccinated babies (2).

The choice of currently available drugs proved to be efficient for treatment of RSV infection is not wide. At mild forms of the disease symptomatic medications are commonly used. Application of interferon and preparations inducing interferon's synthesis causes intensification of inflammatory processes in respiratory tract, and damaging effect of these preparations at RSVI is more clearly expressed than at influenza (4). Ribavirin, an antiviral preparation with wide spectrum of action used for treatment of this infection, had a strong antiviral effect against RSV *in-vitro*, however was not efficient enough for treatment of RSV in babies of the first year of life and, besides, proved to be highly toxic. The preparations of monoclonal antibodies applied for RSV infection treatment are quite expensive and not always efficient.

All above said, search of new drugs inhibiting reproduction of RSV is an urgent task.

At present, Fullerene - (tri-amino-caproic acid) hydrate, a compound produced by ZAO Intelpharm and demonstrated to have antiviral activity against influenza A and B viruses in cell culture and in animal models, is at the stage of nonclinical testing. The compound is also active against some other viruses.

The objective was to study antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate against respiratory-syncytial virus in HEp-2 cell culture. The tasks of the study were as follows**:**

1. Study cytotoxic effect of Fullerene - (tri-amino-caproic acid) hydrate and trisamine (1:1) on HEp-2 cell culture.
2. Study antiviral effect of Fullerene - (tri-amino-caproic acid) hydrate and trisamine (1:1) at various virus doses.
3. Study antiviral effects of Fullerene - (tri-amino-caproic acid) hydrate and trisamine (1:1) at different schemes of the substance administration.

MATERIALS AND METHODS

1. Test substance preparation

Add 5 ml of sterile water to 5 mg of Fullerene - (tri-amino-caproic acid) hydrate substance and 5 mg of trisamine and keep at 37^0C for 30 min up to complete dissolving. Add 5 ml of sterile water to 5 mg of ribavirin substance. Of the obtained solutions with concentration 1 mg/ml make the required concentrations of the preparations (1- 100 μg/ml) on the culture medium. The substances are weighed with accuracy to 0.1 mg on analytical balance.

2. Viruses and cells

Monolayer re-inoculated epithelial cells of human larynx adenocarcinoma HEp-2 grown on DMEM medium with addition of calf serum and antibiotics. Respiratory-syncytial virus (RSV), *Long* strain, was purchased from American Collection of Tissue Cultures (ATTC).

3. **Assessment of cytotoxic effect of Fullerene - (tri-amino-caproic acid) hydrate substance in cell culture**

HEp-2 cells were plated on 96-well Corning-Costar plates, average density 3000 cells/well, and grown in DMEM medium in the presence of 10% calf serum and antibiotics for 24 hours until a complete monolayer was formed. Then the growth medium was removed and in the wells 200 μl of the preparation samples were added in different concentrations in working medium (WM) (DMEM and F12 1:1) with 2% calf serum. The cells were incubated with preparation during 3 days (37^OC, 5% CO_2) and then the cell monolayer was visually examined under inverted microscope. Then the contents of wells were removed and in each well 160 μl of DMEM medium without phenol red with 2% calf serum and 40 μl of tetrazolium MTT dye

(Thiazole blue) in concentration 4 mg/ml, which intensity of staining reflects the level of cell viability, were added. The cells were incubated with the dye during 3 hours (37^{O}C, 5% CO_2). After incubation the content of wells was removed, and in each well 200 µl of dimethyl sulphoxide (DMSO) were added, placed on a shaker for 10 min and in thermostat (37°C) for 10 min to achieve a uniform dye dissolving. Results were counted on automatic colorimeter at wavelengths 540 and 670 nm. The concentration of compounds giving 50% reduction of OD_{540} compared to cell control was considered 50% cytotoxic dose (CTD_{50}).

Assessment of Fullerene - (tri-amino-caproic acid) hydrate substance antiviral activity in cell culture

HEp-2 cells were plated on 96-well Corning-Costar plates, average density 3000 cells/well and grown in DMEM medium in the presence of 10% calf serum and antibiotics for 24 hours until a complete monolayer was formed. Then the growth medium was removed and in cell culture dilutions of the preparation and virus in working medium were added (DMEM and F12 1:1) with 2% calf serum. In the experiments two multiplicities of virus infection (0.1 and 00.2 MOI per cell) and 3 schemes of the preparation administration: 2 hours before infection (prophylactic scheme), simultaneously with infection (therapeutic-prophylactic scheme) and in 2 hours after infection (therapeutic scheme) were used. Compound dilutions – 1-20 µg/ml.

Cell culture with virus and preparation was incubated during 3 days (37^{O}C, 5% CO_2) and then the cell monolayer was visually examined under inverted microscope. Then the substances were removed from wells, and in each well 160 µl of DMEM medium without phenol red with 2% calf serum and 40 µl of tetrazolium MTT dye (Thiazole blue) in concentration 4 mg/ml, which intensity of staining reflects cell viability and characterizes antiviral properties of the preparation, were added. The cells were incubated with the dye during 3 hours (37^{O}C, 5% CO_2). After incubation the content of wells was removed and

in each 200 µl of DMSO were added, placed on a shaker for 10 min, and in thermostat (37°C) for 10 min to achieve a uniform dissolving of the dye. Results were counted on automatic colorimeter at wavelengths 540 and 670 nm. Positive control - the wells not infected with virus, negative control - infected with virus without preparation. For one point of the experiment 4 wells of the plate were used; after calculation of average values a standard deviation was calculated. Semimaximal inhibiting concentration of the preparation (IC_{50}) – the concentration of the preparation giving 50% decrease in the value of optical density - was calculated using GraphPad Prism sofware.

RESULTS

1. Study of cytotoxic effect of Fullerene - (tri-amino-caproic acid) hydrate substance in HEp-2 cell culture

Based on the data from the conducted studies, for assessment of Fullerene - (tri-amino-caproic acid) hydrate substance cytotoxic effect in HEp-2 cell culture the following concentrations of the preparation were chosen: 1, 3, 5, 7,5, 10, 20, 50 and 100 μg/ml. Reference preparation - ribavirin in the same concentrations. Visual examination of the cell monolayers of both preparations performed under inverted microscope after incubation during 72 hours did not reveal considerable changes at all tested concentrations (up to 100 μg/ml). The MTT assay based on the ability of the dye to restore to dark blue staining by live cells confirmed the data obtained at visual examination of cells. Therefore, CTD_{50} for both Fullerene - (tri-amino-caproic acid) hydrate substance and ribavirin was over 100 μg/ml; consequently, both preparation are nontoxic in concentration 100 μg/ml.

2. Study of antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate substance in HEp-2 cell culture

A. Study of the effects of different concentrations of Fullerene - (tri-amino-caproic acid) hydrate at 2 multiplicities of infection

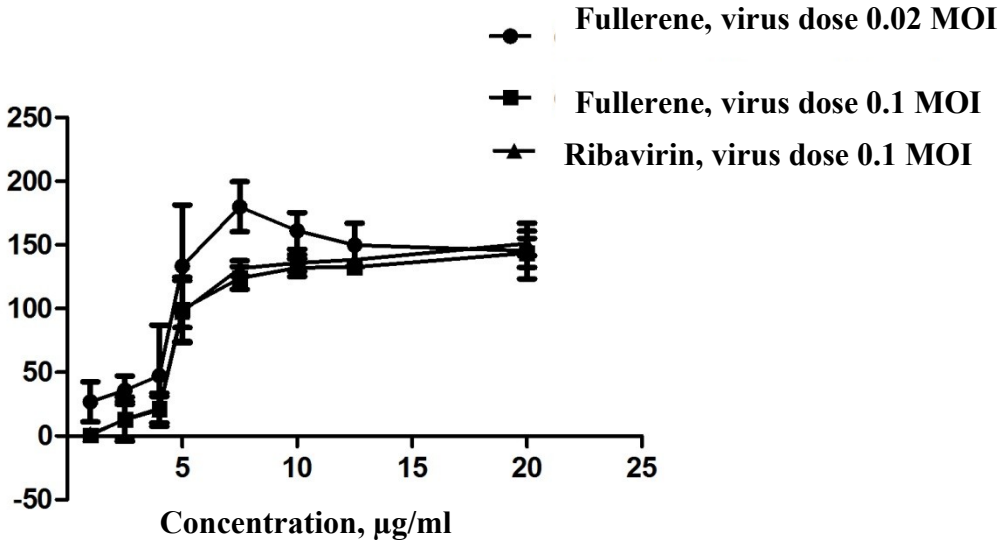
For assessment of antiviral effect of Fullerene - (tri-amino-caproic acid) hydrate substance in HEp-2 cell culture the following concentrations were used: 1, 2,5, 4, 5, 7,5, 10, 12,5 and 20 μg/ml. Reference preparation - ribavirin in the same concentrations.

Antiviral effect of the preparation is in direct proportion to its concentration and in inverse proportion to multiplicity of infection. Our experiments

demonstrated that Fullerene - (tri-amino-caproic acid) hydrate substance efficacy increased with increasing of the substance concentrations and decreased with increasing of multiplicity of infection. Inhibiting was the highest at a higher concentration of the preparations and lower multiplicity of infection. These findings verify virus-specific character of Fullerene - (tri-amino-caproic acid) hydrate substance.

IC_{50} of Fullerene - (tri-amino-caproic acid) hydrate administered 2 hours before infection at multiplicity of infection 0.1 and 0.02 MOI/cell was 5.376±1.39 μg/ml and 3.238±2.02 μg/ml, respectively. In the same experiments IC_{50} of Fullerene - (tri-amino-caproic acid) hydrate at multiplicity of infection 0.1 MOI/cell was comparable with IC_{50} of ribavirin (5 μg/ml).

Fig. 1 Antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate toward RSV in HEp-2 cell culture at 2 multiplicities of infection, administration 2 hours before infection



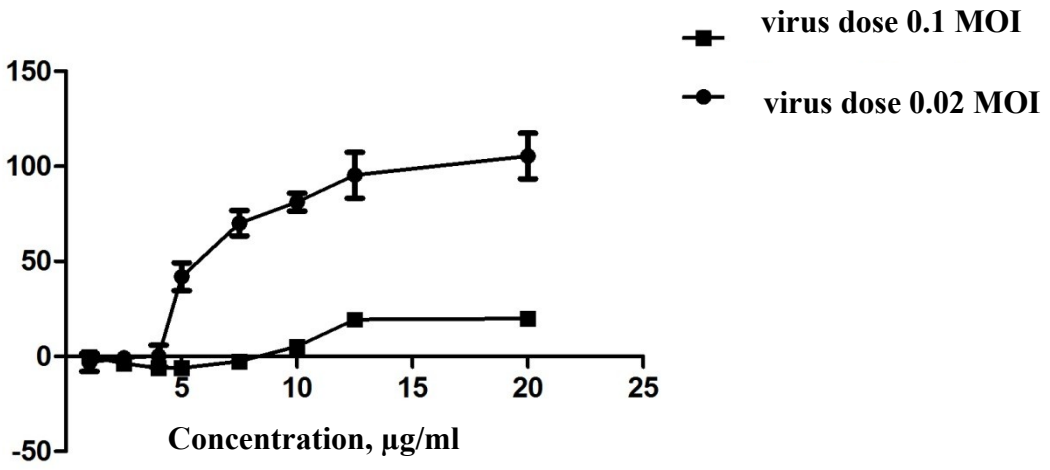
*axis Y – ratio of OD at the given concentration to maximal OD value

B. Study of Fullerene - (tri-amino-caproic acid) hydrate effects at different schemes of administration

The most efficient was prophylactic (2 hours before infection) and therpautic-prophylactic (simultaneously with infection) administration of Fullerene - (tri-amino-caproic acid) hydrate. Administration in 2 hours after infection at virus dose 0.1 MOI was ineffective and semimaximal inhibiting concentration (IC_{50}) was not within the range of the tested concentrations.

IC_{50} at virus dose 0.02 MOI administered in 2 hours after infection was 11.68±3.0 µg/ml.

Fig. 2. Antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate toward RSV in HEp-2 cell culture at 2 multiplicities of infection (administration in 2 hours after infection)



IC_{50} at virus dose 0.02 MOI was at the preparation administration 2 hours before infection 3.238±2.02 µg/ml, when simultaneously with infection - 1.575±1.19 µg/ml, in 2 hours after infection -11.68±3.0 µg/ml.

Fig. 3. Antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate toward RSV in HEp-2 cell culture at different schemes of the preparation administration (virus dose 0.02 MOI)

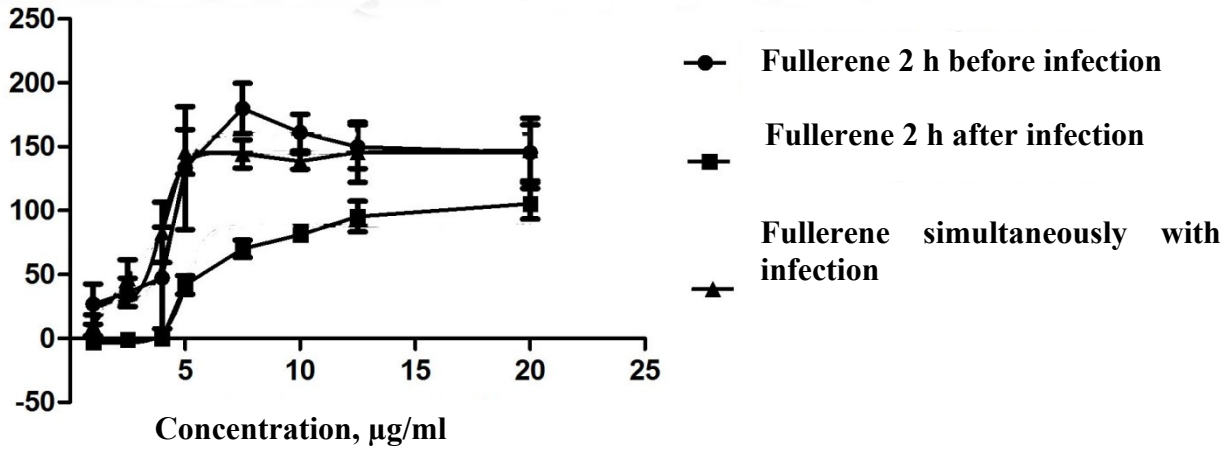


Table 1

Study of Fullerene - (tri-amino-caproic acid) hydrate substance effect on RSV in HEp-2cell culture

Preparations	**CTD50 µg/ml**	**IC_{50} µg/ml**			
		2 hours before infection		**2 hours after infection**	
		0.1 MOI/cell	0.02 MOI/ cell	0.1 MOI/cel l	0.02 MOI/cell
Fullerene - (tri-amino-caproic acid) hydrate	>100	5.376±1.39	3.238±2.02	>20	11.68±3.0 µg/ml
Ribavirin	>100	5.0	n/d	n/d	n/d

CONCLUSION

1. Fullerene - (tri-amino-caproic acid) hydrate substance in concentrations up to 100 μg/ml is nontoxic in HEp-2 cell culture.

2. Fullerene - (tri-amino-caproic acid) hydrate substance efficiently inhibited RSV replication in HEp-2cell culture; its antiviral activity increased with increasing the substance concentration and decreased with increasing the virus dose.

3. Antiviral activity of Fullerene - (tri-amino-caproic acid) hydrate substance toward RSV in HEp-2 cell culture was dependent on the scheme of the substance application. The substance demonstrated antiviral activity at administration simultaneously with infection (IC_{50} 1.575±1.19 μg/ml) and 2 hours before the infection with virus (IC_{50} 3.238±2.02 μg/ml). Administration of the preparation in 2 hours after infection did not have effect on RSV replication.

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