I. N. Falynskova[1], K. S. Ionova[2], A. V. Dedova[3], I. A. Leneva[1], N. R. Makhmudova[1], and L. D. Rasnetsov[3]

ANTIVIRAL ACTIVITY OF FULLERENE-(TRIS-AMINOCAPROIC ACID) HYDRATE AGAINST RESPIRATORY SYNCYTIAL VIRUS IN HEp-2 CELL CULTURE

1 Mechnikov Institute of Vaccines and Sera, Moscow, 105054 Russia.
2 Ivanovskii Institute of Virology, Russian Academy of Medical Sciences, Moscow, 123098 Russia.
3 INTELFARM Company, Moscow, Russia.

The article describes synthesis of fullerene (*tris*-aminocaproic acid) hydrate (FTACA hydrate). The study of FTACA hydrate in HEp-2 cells showed that the compound in non-toxic concentrations (up to 100 μg/mL) has antiviral activity against respiratory syncytial virus. Antiviral activity increases with the increase in the concentration of the substance and decreases when the viral dose is increased, while at the same time inhibitory concentration (IC_{50}) of FTACA hydrate was 5.376±1.39 and 3.238±2.02 μg/ml with the multiplicity of infection equal to 0.1 and 0 .02 MOI respectively. The substance had antiviral activity when added 2 hours prior or at the same time with the infection, while adding it 2 hours after the infection did not have any effect on the reproduction of RSV.

Key words: respiratory syncytial virus; cytotoxicity; antiviral activity; fullerene (*tris*-aminocaproic acid) hydrate.

Respiratory syncytial virus (RSV) is a virus that causes infections of respiratory tract. This infection is most dangerous for young children, 70% of children have respiratory syncytial infection (RSI) in the first year of life and almost every child is infected during the first 3 years. Mortality from complications of the disease caused by this virus in children at the age of 6 weeks to 6 months is one of the highest among infectious diseases. In addition, a severe course of infection occurs in elderly persons with cardio-pulmonological diseases, immunodeficiency states and organ transplants. The immunity formed due to RSI is short and does not provide complete protection against viruses, which is the cause of recurrent infections observed throughout life [1]. Maternal RSV-specific antibodies contained in the blood of newborns, do not guarantee protection against infection, either. The mechanisms of antiviral protection are different in the case of primary and repeated infection which makes the use of an inactivated vaccine ineffective. On the contrary, the administration of this vaccine led to a more severe course of the disease in vaccinated children compared to unvaccinated infants [2]. For the treatment of mild forms of the disease caused by RSV, symptomatic agents are used. The use of interferons, as well as drugs, inducing their synthesis, caused aggravation of inflammatory processes in the respiratory tract [3]. Monoclonal antibodies used for the treatment of RSV infection are expensive and are not always effective. Ribavirin effectively suppresses the reproduction of a wide spectrum of viruses in cell culture, including RSV [4]. However, data of clinical trials do not provide a clear answer to the question concerning its effectiveness against RSV, besides, this preparation has serious mutagenic and teratogenic side effects [5]. In connection with the above-mentioned, the research into new agents that inhibit the production of RSV is, no doubt, currently topical.

Presently, fullerene (*tris*-aminocaproic acid) hydrate (FTACA hydrate), produced by ZAO Intelpharm, is at the stage of preclinical study (Figure 1) [6].

It was previously shown that water-soluble compounds of fullerenes and their amino acids derivatives inhibit the replication of HIV [7] and cytomegalovirus [8], while insoluble derivatives show antiviral activity against the Les Semlika virus and the vesicular stomatitis virus [9]. The purpose of our study is to evaluate the antiviral activity of FTACA hydrate against RSV in the culture of HEp-2 cells. According to the stated goal, the tasks of the study included determination of cytotoxic and antiviral effect of FTACA hydrate against RSV in HEp-2 cells.

Experimental Chemistry

For the studies we used: fullerene C60 ("Fulleren-center", Nizhny Novgorod), *o*-dichlorobenzene (Merk), methyl ether of polyethylene glycol 500 (Merk) without additional purification. Potassium salt of aminocaproic acid was obtained using reaction between ε-aminocaproic acid and KOH in alcohol solution. Electron spectra of N-fullerene (*tris*-aminocaproic acid) were obtained using a Perkin-ElmerLambda 25 spectrometer in an aqueous 0.1 N solution of trisamine. IR spectra of absorption of N-fullerene (*tris*-aminocaproic acid) were obtained using an IR PSM Fourier 1201 spectrometer in the range 400 – 4000 cm^{-1} as solid dispersion of substances in KBr tablets. Thermogravimetric analysis was performed on a Pyris 6 TGA device in nitrogen atmosphere. The elemental C, H, N analysis was carried out using an EurEA 300 microanalyzer. The data of the elemental analysis correspond to the calculated ones.

FTACA Hydrate Synthesis. To a solution of 60 g (0.08 mol) of fullerene C60 in 3.5 l of *o*-dichlorobenzene we added 270 g (1.6 mol) of finely ground anhydrous potassium salt of ε-aminocaproic acid. To the resulting suspension, stirring and heating it no hotter than 60°C, we then added during 2 hours a mixture of 1 liter of *o*-dichlorobenzene and 1.5 liter of polyglycol methyl ether 500. The reaction mix was then stirred at 60°C for 5 hours until complete discoloration of the solution and the formation of a solid precipitate. The precipitate was then filtered, and washed with several portions of hexane and dried in vacuum at 60°C. The isolated mixture of potassium salts of N-fullerene aminocaproic acid and the initial aminocaproic acid were dissolved in 1 liter of distilled water. Then we slowly added 0.1H hydrochloric acid solution while stirring it to achieve pH5.1. The mixture was left until complete settling of the product, the residue was then centrifuged and washed with water to reach pH 6. The residue was dried at 60°C in a vacuum dryer. After that 115 g of N-fullerene (*tris*-aminocaproic acid) hydrate was separated. The yield of the product was quantitative according to the use of fullerene in the reaction. The product was characterized by the methods of thermogravimetry, elemental analysis, UV- and IR-spectroscopy. The data of the elemental analysis correspond to the molecular formula $C_{78}H_{39}O_6N_3 \cdot 10H_2O$. The elemental analysis data for the Ag-salt of the FTACA hydrate correspond to the molecular formula $C_{78}H_{36}O_6N_3Ag_3(10H_2O)$.

The confirmation of the structure of the FTACA hydrate as a tribasic acid, is the Ag-salt formation. The number of carboxyl groups of amino acid fragments attached to the fulleren were determined using reactions with metal salts. Complexes of the composition $C60(H)_3\{NH(CH_2)_5COOAg\}^3 \cdot (10H_2O)$ were separated using the reaction with silver nitrate. The composition of the resulting compound corresponds to the ratio of 1:3 of fullerene to the attached amino acid. The compound contains water molecules on the fullerene sphere and carboxyl groups of amino acid ligands. The amount of hydrated water is determined thermogravimetrically. The substance was dissolved in the mixture of DMSO – water (1:10), DMF – water, and ethanolamine. Since the obtained derivative of fullerene is a strongly associated the system, the electron spectra do not have pronounced absorption bands and represent monotonically falling curves with pronounced transients in the region of 260 nm ($\varepsilon = 5.310^4$), which can be considered as the absorption band of the fullerene component, like polyene. The IR-spectrum of the FTACA hydrate contained absorption bands, which made it possible to make a conclusion about the acid-associated form of N-substituted aminocaproic acid.

Experimental Biology

The substances and solution preparation. To 5 mg of the FTACA hydrate and 5 mg of trisamine (needed to dissolve the FTACA hydrate) 5 ml of sterile water was added and left at 37°C for 30 minutes until complete dissolution. To 5 mg of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazol-3-carboxamide, Sigma-Aldrich, catalog No. R9644) 5 ml of sterile water was added. The obtained solutions with the concentration of 1 mg/ml were used to prepare the necessary amount of preparations for research in the used culture medium.

Viruses and cells. In the experiments, we used single-layered grafted epithelial cells of human laryngeal adeno carcinomas HEp-2, grown in the DMEM (Paneco, RF) medium with the addition of

calf serum and antibiotics. Respiratory syncytial virus (RSV), the Long strain, was acquired in the American tissue cultures collection (ATCC).

Cytotoxic action of FTACA hydrate in cell culture. HEp-2 cells were planted in 96-well plates with the average density of 3000 cells per well and were cultured in DMEM in the presence of 10% calf serum and antibiotics during 1 day to achieve a complete monolayer. Then, the growth medium was removed and the plates were filled with 200 µL of the samples in various concentrations (from 1 to 100 µg/mL) in the working medium (WM) (DMEM and F12 at 1:1) with the 2% calf serum. The cells were incubated with the preparation for 3 days at 37°C in 5% CO2, then, using an inverted microscope, we visually estimated the state of cell monolayer. After that the contents of all the wells were removed and 160 µl of DMEM without phenol red with 2% calf serum was added to each well, as well as 40 µl of tetrazolium red dye MTT (thiazolyl blue) with the concentration of 4 mg/ml, whose intensive color reflects the viability of cells [10]. The cells were incubated with the dye for 3 hours at 37°C in 5% CO_2. After incubation, the contents of the wells were removed and 200 µL of DMSO (dimethyl sulfoxide) was added to each well. The results were calculated using an automatic colorimeter at the wavelengths of 540 and 670 nm. Compound concentration that reduces the value of OD_{540} by 50% against the control cells, was considered the 50% cytotoxic concentration (CTC_{50}).

Antiviral activity of the FTACA hydrate in cell culture. To the cells, prepared as described above, dilutions of the FTACA hydrate were introduced from 1 to 20µg/mL; the same concentrations of Ribavirin were used as the reference preparation. 2 multiplicities of the virus infection were used (MOI): 0.1 and 0.02 MOI per well; and 3 regimens: 2 hours prior infection (prevention), simultaneously with infection (treatment and prevention) and 2 hours after infection (treatment) with the virus. The infected cell culture was incubated with the preparations for 3 days at 37°C in 5% CO_2. Then, using an inverted microscope, the state of cell monolayer was estimated visually. When signs of cytopathic effect were detected, the contents of all the wells were removed, dyed with MTT, and we calculated the results using an automatic colorimeter as described above. Uninfected wells were used as the positive cell control, while wells with infected cells but without the preparation were used as the negative control. 4 wells of the plate were used for one experiment point; after having calculated the average values, the standard deviation was also calculated. The inhibiting concentration of the preparation (IC_{50}) – the concentration which decreases the optical density value by 50% – was calculated using the GraphPadPrism program.

To study the cytotoxic effect of FTACA hydrate in Hep-2 cell culture, the following concentrations of the preparation were selected: 1, 3, 5, 7.5, 10, 20, 50 and 100µg/mL. After the 72 hour incubation, visual estimation showed no significant changes in the cell monolayer in all the test concentrations up to 100µg/mL of FTACA hydrate while 50% of the cell monolayer treated with ribovirin was damaged at 100µg/mL. Dying with MTT confirmed the data obtained by visual examination of the cells. So, CTC_{50} for FTACA hydrate in a cell culture is higher that the concentration of 100µg/mL which proves its low toxicity and allows continuing its further studies in cell culture. For ribavirin the same value was 100µg/mL.

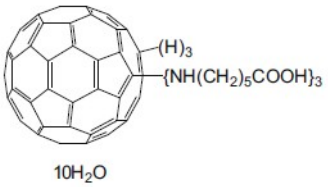

Fig. 1. N-fullerene(*tris*-aminocaproic acid) hydrate.

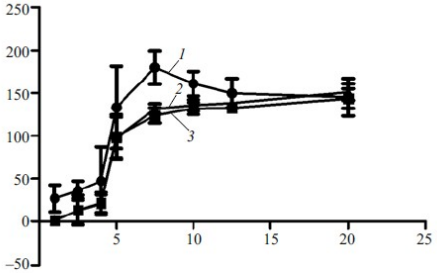

Fig. 2. Antiviral activity of FTACA hydrate against RSV in Hep-2 cell culture at 2 multiplicities of infection adding 2 hours prior to infection: 1 – FTACA hydrate, viral concentration 0.02 MOI; 2- FTACA hydrate, viral concentration 0.1 MOI; ribavirin, viral concentration 0.1 MOI. In figures 2, as well as fig. 3 and 4, the Y axis shows the percentage ratio of the optical density value in a given concentration to the average value of optical density in the cell control; the X axis shows concentration, μg/mL.

The first series of experiments included study of various concentrations of FTACA hydrate for 2 multiplicities of infection.

To evaluate the antiviral activity of FTACA hydrate in Hep-2 culture the following concentrations of the preparation were selected: 1; 2.5; 4; 5; 7.5; 10; 12.5 and 20 μg/mL. The experiments showed that the compound is effective and specifically inhibits the reproduction of RSV in Hep-2 cell culture because the antiviral activity of FTACA hydrate increased along with the increase in its concentration and went down with the increased multiplicity of infection. The highest inhibition was achieved at the highest concentration of the preparation and lowest multiplicity of infection. IC_{50} of FTACA hydrate when administered 2 hours prior infection with the multiplicity of infection equal 0.1 and 0.02 MOI was 5.376±1.39 and 3.238±2.02 μg/mL respectively. IC_{50} of ribavirin at the same conditions was 5±1.6μg/mL (Fig. 2).

The efficacy of viral reproduction inhibition (at 0.1 MOI) of ribavirin and FTACA hydrate was evaluated by two-way analysis of variance with a further introduction of the Bonferroni adjustment. Statistically significant differences were not found at any concentration.

During the following series of studies we studied the activity of the substance against RSV replication depending on the time when it was added to the infected cell culture at various multiplicities of infection. When the preparation was added 2 hours after infection with high multiplicity of infection (0.1 MOI), its activity was low and did not have any significant effect on the replication of the virus (Fig. 3). At such conditions of the experiment, IC_{50} was not in the range of the studied concentrations. However, when the viral concentration was decreased to 0.02 MOI and the preparation was administered 2 hours after, IC_{50} was 11.68±3.0 μg/mL. IC_{50} of ribavirin at the same conditions was 6.1±1.3 μg/mL.

The evaluation of various regimens showed that the highest efficacy was when FTACA hydrate was added preventively (2 hours before infection) and simultaneously with infection.

IC_{50} at the viral concentration of 0.02 MOI was: when the preparation was added 2 hours prior infection – 3.23±2.02 μg/mL, simultaneously with infection – 1.575±1.19 μg/mL (Fig. 4).

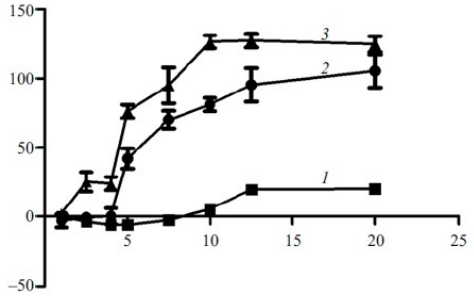

Fig. 3. Antiviral activity of FTACA hydrate against RSV in Hep-2 cell culture at 2 miltiplicities of infection when added 2 hours after infection: 1 – FTACA hydrate, viral concentration 0.1 MOI; 2 – FTACA hydrate, viral concentration 0.02 MOI; 3 – ribavirin, viral concentration 0.1 MOI.

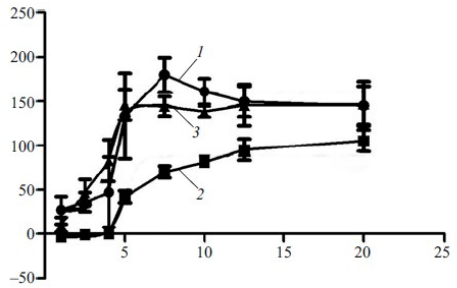

Fig. 4. Antiviral activity of FTACA hydrate against RSV in Hep-2 cell culture at various regimens and the viral concentration of 0.02 MOI: 1 – 2 hours prior infection; 2 – 2 hours after infection; 3 – simultaneously with infection.

The evaluations show that FTACA hydrate in HEp-2 cell culture has specific antiviral activity against respiratory syncytial virus which increases when the substance concentration increases and decreases when the viral concentration increases. The substance has the highest effect when administered simultaneously with infection and 2 hours prior to infection; at the latter conditions its activity is comparable with that of ribavirin. The selectivity index was calculated using the obtained data on the substance cytotoxicity in the same cell culture, and it exceeds 20 while for ribavirin it equals 20. It is important to note that the data obtained by us had been confirmed in the studies performed by National Health Institutes (Antiviral Research Institute, Uta, USA) (personal data of ZAO Intelpharm). The studies, performed in Hep-2 cell culture with a different RSV strain (A), the IC_{50} and selectivity index for FTACA hydrate were 2.04 and 48 μg/mL respectively. To identify the CPA, the American researchers used a different stain of viable cells with Cell Titer-Glo (Promega) (Intelpharm's files).

Considering the totality of our evaluations, as well as the previously obtained data on the antiviral activity of FTACA hydrate against other viruses, further studies of FTACA hydrate are deemed reasonable to develop and create a new antiviral preparation.

LITERATURE

1. A. R. Murry, S. F. Dowell, *Hosp. Pract.*, **32**(7), 87 – 88, 91 – 94 (1997).
2. H. W. Kim, J. G. Canchola, C. D. Brandt, et al., *Am. J. Epidemiol.*, **89**(4), 442 – 434 (1969).
3. M. S. Boukhvalova, G. A. Prince, J. C. Blanco,*Virol. J.*,**7**, 7 –20 (2010).
4. J. F. Hruska, J. M. Bernstein, R. G. Douglas, et al., *Antimicrob. Agents Chemother.*,**17**(5), 770 – 775 (1980).
5. P. R. Wyde,*Antiviral. Res.*,**39**(2), 63 – 79 (1998).
6. L.D. Rasnetsov, Ya. Yu Shwarzman, O.N. Souvorova, Hydrated n-fullerene-amino acids, the method of obtaining them and pharmaceutical compositions based on them, PatentRF RU2458046C1, bulletin of inventions No. 22 (2012).
7. R. F. Schinazi, R. Sijbesma, G. Srdanov, et al., *Antimicrob. Agents Chemother.*,**8**, 1707 – 1710 (1993).
8. R. A. Kotelnikova, G. N. Bogdanov, E. C. Frog, et al., *J. Nanoparticle Res.*,**5**, 561 – 566 (2003).
9. F. Kaesermann, C. Kempf, *Antiviral Res.*, **34**, 65 – 70 (1997).
10. T. Mosmann, *J. Immunol. Methods*, **65** (1 – 2), 55 – 63 (1983).

ANTIVIRAL ACTIVITY OF FULLERENE-(TRIS-AMINOCAPROIC ACID) HYDRATE AGAINST RESPIRATORY SYNCYTIAL VIRUS IN HEp-2 CELL CULTURE

I. N. Falynskova[1], K. S. Ionova[2], A. V. Dedova[3], I. A. Leneva[1], N. R. Makhmudova[1], and L. D. Rasnetsov[3]
[1] Mechnikov Institute of Vaccines and Sera, Moscow, 105054 Russia.
[2] Ivanovskii Institute of Virology, Russian Academy of Medical Sciences, Moscow, 123098 Russia.
[3] INTELFARM Company, Moscow, Russia.

The synthesis of fullerene-(tris-aminocaproic acid) hydrate (FTACAH) is described. The study of FTACAH in HEp-2 cell culture showed that this compound in not-toxic concentrations (up to 100 μg/mL) inhibited the reproduction of respiratory syncytial virus (RSV). The inhibitory effect of FTACAH increased with the drug concentration and decreased with increasing virus dose (multiplicity of infection, MOI). The IC_{50} (50% inhibitory concentration) ranged from 5.37±1.39 μg/mL to 3.238 ±2.02 μg/mL at MOI = 0.1 and 0.02, respectively. The study of drug administration regimes showed that the maximum inhibiting effect was observed when FTACAH was added 2 h before infection or simultaneously with infection. No any inhibitory effect on RSV replication in HEp-2 cell culture was observed when FTACAH was added 2 h after infection.

Keywords: respiratory syncytial virus (RSV); cytotoxicity; antiviral activity; fullerene-(tris-aminocaproic acid) hydrate