

УДК 578.826:615.281

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ANTIADENOVIRAL ACTIVITY OF FLUORIDE-CONTAINING NUCLEOSIDES AND BISPHTHONATES DERIVATES

*Human adenoviruses cause various acute diseases including gastrointestinal and respiratory disorders. However, there are no clinically approved specific anti-adenoviral drugs. Therefore, the search of drugs and regimens that would be effective, safe for prolonged use, and available at a cost to a wide range of patients is extremely important. **The aim of the work** was to study the cytotoxicity and antiviral activity of six new fluorochemical compounds with respect to human adenovirus serotype 5 in vitro. **Methods.** Cytotoxicity of the compounds was determined by MTT-test. The lysosomal activity of cells was estimated using neutral red dye. Cytomorphological method was used to identify adenovirus infected cells containing specific virus inclusion. In addition, the anti-adenoviral activity of the most effective compounds was confirmed by real-time PCR analysis. **Results.** CC_{50} index measured by MTT- test, ranged from 125 mg/ml to 1000 μ g/ml. CC_{50} index determined with neutral red dye ranged from 630 μ g/ml to 2500 μ g/ml. It was discovered that the toxicity of compounds dependent on their solubility. The anti-adenoviral activity was shown for three compounds referred to G22, G26 and 10S-23 with EC_{50} values of 60, 120 and 90 μ g/ml, respectively. PCR analysis also revealed anti-adenoviral activity of the compounds G26 and 10S-23. **Conclusions.** The analysis of the cytotoxic and antiviral activity of six new fluorochemical compounds was conducted. Cytomorphological analysis showed the antiviral activity against adenovirus serotype 5 for the compounds G22, G26 and 10S-23. Using PCR analysis, the anti-adenoviral activity of the compounds G26 and 10S-23 was demonstrated.*

Key words: adenovirus, fluoride-containing compounds, cytotoxicity, antiviral activity.

More than 60 serotypes of human adenoviruses, which cause a variety of course and severity of clinical signs of infectious disease are known. Particularly high risk of adenovirus infection is observed in recipients after transplantation of stem cells, and in people with immune deficiencies, particularly those receiving immunosuppressive therapy following organ transplantation or HIV-infected patients [11].

However, there is no specific drug for the treatment of adenoviral diseases [14, 7, 10]. In clinical practice, the treatment of eye diseases caused by adenovirus includes a number of substances, which possess purely virucidal action and affect the



extracellular virus (oxolinum, tebropheum, bonaphtonum). Ribavirin and cidofovir are the most frequently used for other adenoviral infections. However, their use is ineffective in some cases including disseminated adenovirus infection in persons with immunodeficiency [8, 13, 9, 22, 5]. Furthermore, cidofovir is characterized by several disadvantages including the renal toxicity, irritant effect in the adenoviral diseases of the eye, the appearance of resistant strains. In addition, several publications describe the insensitivity of some adenovirus serotypes to ribavirin [16, 18].

Recently published data describes a new drug referred to brincidofovir, a lipid conjugate of cidofovir, that show inhibiting effect on adenovirus infection. However, unlike cidofovir the new drug is much more efficient and safer due to the lipid components (flippases) that accelerate the entry and accumulation of the drug in the cell enhancing its bioavailability. At present, a phase III multicenter study of brincidofovir is recruiting participants to study its efficacy in the treatment of HAdV [21, 19].

Therefore, the search of drugs and regimens that would be effective, safe for prolonged use, and available at a cost to a wide range of patients is extremely important.

The studying of fluorinated nucleoside sugars chemistry became the basis for the development of promising chemotherapeutic agents with antitumor and antiviral effects. Based on the purine and pyrimidine nucleotide analogues and fluorinated heterocycle molecules a number of new generation drugs with anticancer effect were developed. Thus, fludarabine phosphate is an effective anticancer compound for the treatment of acute or chronic lymphocytic leukemia and non-Hodgkin's lymphomas. The synthesis and implementation into clinical practice of 5-fluorouracil analogue was an extremely important achievement of modern medicinal chemistry. This compound is now widely used in the treatment of malignant tumors of various organs. It is known that 3'-deoxy-3'-fluoro-D-deoxyribonucleosides act as inhibitors of several DNA- and RNA-containing viruses. For example, 3'-deoxy-3'-fluoroadenosine inhibits the replication of different RNA-containing viruses including poliovirus, Coxsackie virus, Sindbis virus, and DNA-containing cowpox virus. Some pyrimidine ribonucleosides have antiviral activity against herpes simplex virus. For instance, 2'-deoxy-2'-fluorocytidine is a strong and selective inhibitor of HCV RNA polymerase [6, 12].

The aim of the work was to study the cytotoxicity and antiviral activity of some new fluorochemical compounds synthesized at the Institute of Organic Chemistry NAS Ukraine, with respect to human adenovirus serotype 5 *in vitro*.

Materials and methods

Inoculated cell culture including MDBK (bovine kidney) and Hep-2 (larynx epidermoid carcinoma) were used. Cells were grown in a medium consisting of 45% DMEM (BioTestMed, Ukraine), 45% RPMI 1640 (Sigma, USA), 10% serum of cattle inactivated by heating (Sigma, USA), and antibiotics penicillin (100 µg/ml) and streptomycin (100 µg/ml). The cultivation of cells was performed according to standard procedure [3]. The reference strain of human adenovirus serotype 5 was



obtained from the collection of the Institute of Microbiology, Medical University of Budapest. The virus was accumulated in cell culture Hep-2, titrated and stored at a temperature of -20°C .

Test compounds. G22 and G23 – nucleosides modified on carbohydrate part (2-N-substituted-4-tosyl-5-polyfluoroalkyl-1,2,3-triazole); G26 and G27 – fluorine containing nucleosides based on uracil; 10S-23 and 10S-24 – sodium salt of N-(2,2,2-trifluoroethyl) phenylalanine and N-(2,2,3,3-tetrafluoropropionyl) phenylalanine (Fig. 1). An official drug ganciclovir (“CYMEVENE” Roche, Switzerland) (Fig. 1) was used as a reference drug.

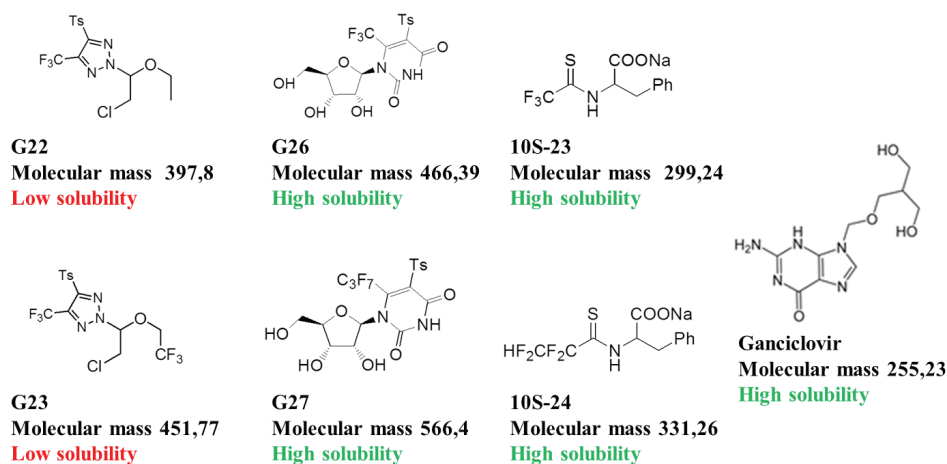


Fig. 1. Test compounds

The compounds were dissolved in dimethylsulfoxide (DMSO, Sigma, USA) to concentration of 20 mg/ml and stored at 4°C . Working twofold serial dilutions from 1 mg/ml to $8\ \mu\text{g/ml}$ were prepared in the medium for cell culture (RPMI-1640, Sigma, USA) without serum immediately before use. Solutions were sterilized using syringe filtration through membrane filters with pore diameter of 0,22 microns (Sarstedt, Germany).

Cytotoxicity of the compounds was determined by MTT-test according to the standard protocol [17]. The lysosomal activity of cells was estimated using neutral red dye [20]. The results of colorimetric analysis were recorded using Multiscan FC device (Thermo Fisher Scientific, USA) at a wavelength of 540 nm. The concentrations of substances that inhibit 50% of cell viability compared to control cells (CC_{50}) were measured using a linear regression method in Microsoft Excel 10.

Cytomorphological method was used to identify adenovirus infected cells containing specific virus inclusion [2]. MDBK cells were grown in test tubes with stripes covering glasses. Then, cells were infected with the virus of plurality 10 ICU/cell. After 1,5 hours of virus adsorption at room temperature cells were washed using Hanks solution. Next, experimental substances of different concentration dissolved in a supportive medium were added. Each concentration was used in triplication. Adenovirus infected cells were used as control. The experiments were conducted using 2-fold dilutions of the compounds (31, 62, and 125 mg/ml) and time point of



48 hours after infection and compound treatment. The data analysis was performed as described previously [1]. The half maximal effective concentration (EC_{50}) was estimated as the concentration of the compound which induced to 50% of its maximal effectiveness that was observed.

In addition, to confirm the antiviral action of the most effective compounds real-time PCR analysis was used. The adenovirus genome region responsible for the synthesis of late adenovirus structural hexon protein was used as a target. Statistical analysis of the data was performed according to standard approaches with the calculation of statistical error (standard deviation) using Microsoft Excel 2010.

Results and discussion

The research of cytotoxicity of new fluorochemical compounds was conducted on inoculated monolayer cell culture MDBK, which is sensitive to human adenovirus. CC_{50} indexes were estimated using MTT-test and are presented in Figure 2. It was shown that compounds with low solubility in the growth medium significantly greater toxicity. Compounds G22 and G23, which have similar structure and differ only by the presence of additional trifluoromethyl group (CF_3 - group) in compound G23 showed toxic effects. However, CC_{50} index was 250 $\mu\text{g/ml}$ for G22 and 125 $\mu\text{g/ml}$ for compound G23. Therefore, it was suggested that the presence of the CF_3 -group increases the toxicity. Compounds G26 and G27 also differ only in the number of fluorine atoms in the molecules. However, no difference in the bioavailability was detected, whereas both compounds showed low toxic effect and high solubility. CC_{50} indexes for both substances were 630 $\mu\text{g/ml}$. The compounds 10S-23 and 10S-24 demonstrated relatively low rate of cytotoxicity at the maximal concentration used in the study (1000 $\mu\text{g/ml}$). Therefore, using the method of statistical analysis (function prediction in Microsoft Excel 10) CC_{50} indexes for these compounds were determined (1250 and 1700 $\mu\text{g/ml}$, respectively). Their high solubility is caused by the presence of polar groups ($-NH$, $-COO$), which can form hydrogen bonds with the solvent increasing the solubility of the compound. Reference drug ganciclovir showed high solubility and increased rates of cytotoxicity with CC_{50} value greater than 1000 $\mu\text{g/ml}$ (1200 $\mu\text{g/ml}$).

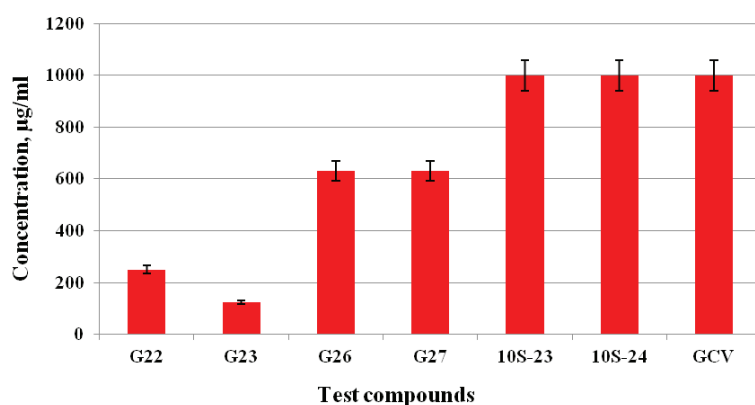
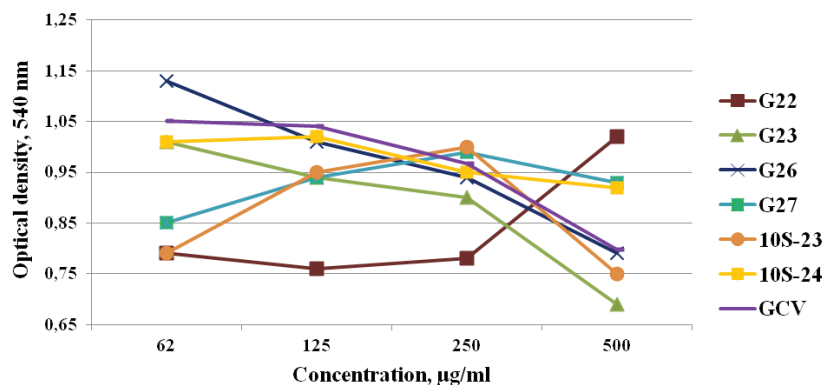


Fig. 2. CC_{50} indexes of compounds identified by MTT method

Therefore, the higher CC_{50} indexes of compounds and, as a result, their lower toxicity are likely related to their solubility in the medium. Moreover, highly soluble compounds including G26, G27, 10S-23, 10S-24 have low toxicity even at high concentrations.

The influence of these compounds on the lysosomal activity of the cell was analyzed using neutral red dye staining. Lysosomal membranes are well-permeable for the dye only when the cell is alive and fully functioning. The dye is able to accumulate in lysosomal matrix and cannot be washed out with ethanol, allowing the identification of the alive and proliferating cells [20]. Lysosomal activity of the compounds was estimated in the concentration range of 16-500 $\mu\text{g/ml}$ (Fig. 3). Using statistical analysis (function prediction in Microsoft Excel 10) it was found that the rates of lysosomal activity ranged from 630 $\mu\text{g/ml}$ and, in some cases, exceeded the index of 1000 $\mu\text{g/ml}$ (>2500 $\mu\text{g/ml}$ for 10S-24).

Comparing the effects on cell viability estimated by two methods it was suggested that compounds G22 and G23 have different influences on cell compartments. These compounds showed significant inhibitory effect on the functioning of the mitochondria with increased levels of lysosomal activity, indicating the activation of cell death.



Annotation: The optical density of control was 0,85

Fig. 3. The levels of the lysosomal activity determined using neutral red dye staining

For the analysis of antiviral activity of the compounds non-toxic concentrations with the values lower than CC_{50} index were used. Compounds added as part of a growth medium after virus adsorption. Compounds G23 and G27 did not inhibit the reproduction of human adenovirus serotype 5. However, inhibition of adenovirus reproduction was shown for compounds G22, G26 and 10S-23 (Table 1).

Therefore, the compounds G22 and G26 suppressed reproduction of HAdV-5 by 56 % at a concentration of 125 $\mu\text{g/ml}$ and over 30 % at the concentration of 31 $\mu\text{g/ml}$.

Compound 10S-23 suppressed the reproduction of HAdV-5 by 62% and 2% at the concentrations of 125 $\mu\text{g/ml}$ and 31 $\mu\text{g/ml}$, respectively. Compound 10S-24 showed low inhibitory effect on the reproduction of adenovirus (6%) at the concentration of 125 $\mu\text{g/ml}$. The effective antiviral concentrations that cause the reduction of specific



virus inclusions by 50 % (EC_{50}) were 60, 120 and 90 $\mu\text{g/ml}$ for compounds G22, G26 and 10S-23, respectively. The EC_{50} index of the reference compound was 50 $\mu\text{g/ml}$.

Table 1

Antiviral activity of the compounds

Chemical structure of compounds	Compound code	% Inhibition of virus reproduction		
		Concentration, $\mu\text{g/ml}$		
		31	62	125
Nucleosides derivatives	G22	39	52	56
	G23	N/A	N/A	N/A
	G26	30	41	56
	G27	N/A	N/A	N/A
Bisphosphonates derivatives	10S-23	2	42	62
	10S-24	N/A	N/A	6

Annotation: N/A – not active

Compounds that demonstrated significant anti-adenoviral activity were also examined by real-time PCR. Compound G22 showed no suppressive effect on the adenovirus reproduction at analyzed concentrations. Compound G26 inhibited replication of viral DNA by 100% at the concentration of 31 $\mu\text{g/ml}$ and by 27% at the concentration of 125 $\mu\text{g/ml}$. The inhibition of the viral DNA replication was shown for all analyzed concentrations of the compound 10S-23 with the inhibition effect ranging between 27% and 40%, depending on the concentration of the compound. Although, PCR analysis showed that the reference drug ganciclovir caused the inhibition of adenovirus reproduction, the partial replication of viral DNA occurred.

The largest number of drugs used in medical practice is represented by nucleosides with various modifications in the structure. The presence of fluorine atoms in the molecule of nucleoside leads to the changes of its chemical, physical and biological properties [4, 12]. There are already enough fluoride-containing nucleoside drugs used for the treatment of diseases caused by viruses such as Coxsackie virus, poliovirus, HCV and HIV [15]. Therefore, further progress in the synthesis of new fluorochemical compounds will contribute to the understanding of their molecular mechanisms of action.

It is known that the antiviral effect of abnormal nucleosides in most cases is caused by the intracellular phosphorylation of inactive nucleoside and formation of active nucleotide [4]. As nucleotide analogues, they compete with natural nucleotides for the enzymes involved in the synthesis of nucleotides and nucleic acids. Therefore, the incorporation of abnormal nucleosides into the nucleic acids makes them non-functional [18, 6].

Analysis of the compounds cytotoxicity demonstrated that the cytotoxicity correlates with the compound solubility in growth medium. The insoluble compounds



were shown to be toxic for cells. Their soluble components were suggested to affect the integrity of the cell structure increasing the level of cell death.

Determination of the antiviral activity assessed via cytomorphological method and real-time PCR (for the most active compounds) demonstrated the inhibitory effect of the most compounds on the reproduction of the virus. The results of the both approaches confirmed that the compounds reduced the number of viral DNA. Therefore, it can be assumed that the compounds G26 and 10S-23 affected the stage of the viral DNA replication. Inhibition of this phase of the adenovirus reproduction led to the problems with the life cycle progression and infectious particles formation.

Using cytomorphological method the antiviral effect of the compound G22 was shown. However, the antiviral effect of this compound was not confirmed by the PCR suggesting that the compound affects other stages of virus reproduction including mRNA synthesis. Therefore, the compound G22 suppressed further synthesis of viral proteins, disrupting the formation of viral particles. As a result, that caused the absence of the adenovirus specific inclusions in the cell nuclei. However, that did not affect process of the viral genome replication confirmed by the real-time PCR.

The analysis of the cytotoxic and antiviral activity of six new fluorochemical compounds was conducted. It was found that the cytotoxic and antiviral activity of the related compounds depend on the presence or absence of certain chemical groups and a number of fluorine atoms in the molecule. Cytomorphological analysis showed the antiviral activity against adenovirus serotype 5 for the compounds G22, G26 and 10S-23. Using PCR analysis, the anti-adenoviral activity of the compounds G26 and 10S-23 was demonstrated.

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ВИВЧЕННЯ АНТИАДЕНОВІРУСНОЇ ДІЇ ФТОРВМІСНИХ СПЛУК НУКЛЕОЗИДНОЇ ТА НЕНУКЛЕОЗИДНОЇ ПРИРОДИ

Реферат

Мета роботи: дослідження цитотоксичності та антивірусної активності ряду нових фторвмісних сполук відносно аденовірусу людини 5 серотипу в системі *in vitro*. **Методи.** Визначення мітохондріальної активності клітин проводили за допомогою МТТ-методу. Лізосомальну активність клітин досліджували із застосуванням барвника нейтрального червоного. Антивірусну активність речовин досліджували цитоморфологічним методом із застосуванням фарбника акридинового оранжового та за допомогою ПЛР аналізу. **Результати.** Показник CC_{50} встановлений за допомогою МТТ-тесту, коливався від 125 мкг/мл (сполука G23) до >1000 мкг/мл (сполуки 10S-23 та 10S-24). CC_{50} визначений з використанням нейтрального червоного мав значення: від 630 мкг/мл до >2500



мкг/мл. Була виявлена залежність токсичності сполук від їх розчинності. Показана антиаденовірусна активність для 3-х сполук – G22, G26 та 10S-23, EC_{50} для них складала 60, 120 і 90 мкг/мл, відповідно. ПЛР аналізом також було виявлено антиаденовірусну дію сполук G26 та 10S-23. **Висновки.** Отже, було проведено дослідження цитотоксичної та антивірусної дії 6 нових фторвмісних сполук. Виявлено, що у споріднених сполук, що відрізнялись кількістю атомів фтору у молекулі змінювалась їх цитотоксичність та антивірусна дія. Цитоморфологічним методом показано антивірусну активність відносно аденовірусу 5 серотипу сполук G22, G26 та 10S-23. З використанням ПЛР аналізу також встановлено антивірусну дію сполук G26 та 10S-23.

Ключові слова: аденовірус, фторвмісні сполуки, цитотоксичність, антивірусна активність.

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ИССЛЕДОВАНИЕ АНТИАДЕНОВИРУСНОГО ДЕЙСТВИЯ ФТОРСОДЕРЖАЩИХ СОЕДИНЕНИЙ НУКЛЕОЗИДНОЙ И НЕНУКЛЕОЗИДНОЙ ПРИРОДЫ

Реферат

Цель работы: исследование цитотоксичности и антивирусной активности ряда новых фторсодержащих соединений относительно аденовируса человека 5 серотипа в системе *in vitro*. **Методы.** Определение митохондриальной активности клеток проводили с помощью МТТ-метода. Лизосомальную активность клеток исследовали с применением красителя нейтрального красного. Антивирусную активность веществ определяли цитоморфологическим методом с применением красителя акридинового оранжевого и ПЦР анализа. **Результаты.** Показатель CC_{50} установленный с помощью МТТ-теста, колебался от 125 мкг/мл (соединение G23) до > 1000 мкг/мл (соединения 10S-23 и 10S-24). Показатель CC_{50} определенный с использованием нейтрального красного имел значения: от 630 мкг/мл до >2500 мкг/мл. Была выявлена зависимость токсичности соединений от их растворимости. Показана антиаденовирусная активность для 3-х соединений – G22, G26 и 10S-23, EC_{50} для них составляла 60, 120 и 90 мкг/мл, соответственно. ПЦР анализом также было обнаружено антиаденовирусное действие соединений G26 и 10S-23. **Выводы.** Таким образом, было проведено исследование цитотоксического и антивирусного действия 6 новых фторсодержащих соединений. Виявлено, что в соединениях, которые отличались количеством атомов фтора в молекуле, изменялись показатели их цитотоксичности и антивирусное действие. Цитоморфологическим методом показано антивирусную активность в отношении аденовируса 5 серотипа соединений G22, G26 и 10S-23. С использованием ПЦР анализа также установлено антивирусное действие соединений G26 и 10S-23.

Ключевые слова: аденовирус, фторсодержащие соединения, цитотоксичность, антивирусная активность.



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Стаття надійшла до редакції 09.11.2016 р.

