Viruses and Cell

In vitro and *in vivo* investigations on anti-influenza effect of adamantyl (alkyl, cycloalkyl) derivatives of aminopropanol-2

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Aim. To establish anti-influenza activities for 8 compounds of adamantyl (alkyl, cycloalkyl) derivatives of aminopropanol-2 *in vitro* and in vivo investigations. **Methods.** The antiviral action of compounds was determined *in vitro* by reduction of infectious titer of the influenza virus in the Madin-Darby Canine Kidney (MDCK) cell culture and in vivo on the model of influenza pneumonia in mice. **Results.** Three of eight studied compounds inhibit the reproduction of the influenza virus strain A/FM/1/47(H1N1) by more than 2 lg ID_{50} in the concentration range from < 0.39 µg/ml (the compounds 30 and 33) to 1.56 µg/ml (the compound 5), their chemotherapeutic indices are 256 and 16, respectively. The experiments in vivo showed that in prophylactic scheme the efficiency index (EI) of the compounds 5 and 30 is 60 %; in therapeutic scheme EI of the compounds 5 and 33 is 100 % that is two times higher, than for rimantadine. **Conclusions.** High chemotherapeutic indices of the compounds 30, 33 and their ability to suppress the influenza virus reproduction in the MDCK cell culture by more than 2 lg ID_{50} indicate the anti-influenza activity of these compounds. It has been also confirmed by in vivo experiment.

K e y w o r d s: influenza virus, adamantyl (alkyl, cycloalkyl) derivatives of aminopropanol-2, antiviral activity.

Introduction

For today in the world and in Ukraine in particular, is unstable epidemic situation for many infectious diseases, including viral. According to the WHO estimates by 2030, infection diseases will be one of the major causes of mortality among people (Antimicrobial resistance: global report on surveillance 2014, http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1). Influenza is a leader among the respiratory tract infections by the

number of cases and distribution. Airborne droplets way of distribution of flu infection leads to the rapid spread of the pathogen, and high antigenic variability of the influenza virus results in the appearance of new strains causing epidemics and pandemics. According to the WHO data, the flu and flu-like illnesses affect annually 5 – 30 % of the world population. The flu and acute respiratory viral infections annually affect about 15 million people in Ukraine [1]. Despite the recent achievements in diagnostics and therapy, the problem of treatment of influenza remains a relevant problem, as well as the establishment of effective chemotherapeutic agents for prevention and therapy of this infection. Nowadays the process of creating new medicines commonly starts from finding new drug candidates – structural prototypes of future chemotherapeutic agents with the desired pharmacological effect [2]. The search for antimicrobial compounds is carried out among new chemical classes as well as among new derivatives of already known compounds affecting certain targets. Since discovery of adamantane its derivatives have proven to be the most promising as viral inhibitors [3–6]. The best of them is aminoadamantane (amantadine, rimantadine) [3, 7], which entered the pharmaceutical market and is successfully used as an anti-influenza drug.

The adamantane molecule is highly reactive compared with other hydrocarbons, which allows obtaining a wide range of poly-functional derivatives. The molecule can be modified by the C–H bond activation for the direct nucleophilic substitution or radical reactions [8–12]. It can be also modified by the introduction of substituents or heterocyclic atoms for designing new derivatives [13, 14].

It is known from literature that amino alcohols and the products of their substitution in the amino and hydroxyl groups have polyvalent pharmacological effects (stimulators or blockers of α and β-adrenaline, adrenaline mimetics, antibiotics, *etc.*) [15, 16]. It is also known that adamantane containing amino alcohols demonstrates antiviral action [17]. The modification of β-adrena blockers structures via the introduction of various adamantyl fragments as amino groups results in the appearance of antivirus effects [18]. Based on this knowledge it seemed advisable to study an antiviral effect of new adamantane derivatives containing amino alcohols and their related alkyl and cycloalkyl structural analogs [19, 20].

Materials and Methods

The synthesized compounds

Investigated adamantanyl (alkyl, cycloalkyl) derivatives of aminopropanol-2 were synthesized in Institute of Organic Chemistry of the NAS of Ukraine according to the procedures developed in [15]. The structure and purity of the synthesized compounds were proved via elemental analysis, proton magnetic resonance spectroscopy and chromatography mass spectrometry methods. The common formula of the tested compounds is

$$ROCH_2CH(OH)CH_2R^1$$

where: R – $Ad(CH_2)_2$, R^1 – cyclohexylamin (the compound 5); R – $Ad(CH_2)_2$, R^1 – 1,1,3,3-tetramethylbutylamine (the compound 6); R – tretbutyl, R^1 – 2,2,6,6-tetraethyl piperidin amine (the compound 33); R – allyl, R^1 – 1,1,3,3-tetramethylbutyl-amine (the compound 40); R – tret-amyl, R^1 – 2,2,6,6-tetramethyl-4-hydroxy piperidine amine (the compound

47); R – cyclopentyl, R^1 – 2,2,6,6-tetramethyl-4-hydroxy piperidine amine (the compound 48); R – bornyl, R^1 – 2,2,6,6-tetramethyl-4-hydroxy piperidine amine (the compound 51). The compounds 48 and 51 were investigated in the alkali form.

Depending on the radicals structure all tested compounds were divided into the adamantane containing (compounds 5 and 6), bornyl containing (the compound 51), the compounds with cyclic substituents in alkoxy group (the compounds 30 and 48) and the compounds with alicyclic substituents in alkoxy group (the compounds 33, 40, 47). All synthesized compounds are colorless and odorless crystalline substances with different solubility in water and 96 % alcohol. The 1 mg/ml matrix solutions of the studied compounds were prepared using a medium for cell cultures without serum. These solutions were sterilized via filtration through cellulose filter with 0.22 μm pore diameter. The solution was stored in sterile bottles at –20 °C before using.

Virus and cell culture

The mouse-adapted influenza virus A/FM/1/47(H1N1) was cultivated in Madin-Darby Canine Kidney (MDCK) cells (ATCC, CCL-34). MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5 % heat inactivated fetal bovine serum (FBS) in 37 °C incubator with 5 % CO_2. The influenza A/FM/1/47(H1N1) and MDCK cells were obtained from the Bank of cell cultures of the laboratory of experimental chemotherapy of viral infections of the L. V. Gromashevsky Institute of epidemiology and infectious diseases of NAMS of Ukraine.

Virological tests in vitro

Anti-influenza activity of the tested compounds was evaluated by chemotherapeutic index (CTI) determined as the ratio of the maximum tolerated dose (MTD) to the minimum active concentration (MAC).

MTD was determined as the greatest concentration of the tested compound that did not cause the cytopathic effect (CPE) in the monolayer of MDCK cells [16, 17]. For this purpose, the growing MDCK cells were plated at a density of 1×10^5 cells/ml and incubated in 96-well plates for 24 h (37 °C, 5 % CO_2). Then the cells were treated with the tested compounds of different concentration (3.12, 6.25, 12.5, 25, 50, 100 μg/ml) for 5 days. The wells containing no compounds were used as a negative control. In the experiments, we used at least ten holes for each dilution of the substance. The CPE was determined by changed cell morphology (degeneration or exfoliation of cells from the hole surface).

MAC of the studied compounds was determined by the degree of reduction of infectious titer of the virus according to the General established procedure [16, 17]. The virus titers were calculated as 50 % of tissue culture infectious dose ($TCID_{50}$) using the Kerber formula (Manual for the virological investigation of polio, http://apps. who.int/iris/bitstream/10665/68762/1/WHO_IVB_04.10.pdf):

$$Lg\ TCID_{50} = L\text{-}d(S\text{-}0.5),$$

where L – the initial dilution, d – the difference between Lg of serial dilutions, S – the sum of the proportions of tested objects, which gave a positive result.

The hemagglutination inhibition test (HAI) was carried out to study the influence of tested compounds on the influenza virus hemagglu-

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tination activity (HA). The tested substances diluted in the range of 50 – 0.36 mkg/ml were incubated with the virus (4 agglutination units) for 30 min at room temperature; 0.75 % erythrocytes suspension of Guinea pigs were added, shaken and left at room temperature for 60 min. As a titer of HAI was taken a limiting dilution, which gives a complete delay of the hemagglutination reaction, that is ++++.

Virological studies in vivo

The anti-influenza activity of tested compounds was studied via simulation of influenza pneumonia in mice using prophylactic and therapeutic schemes of the compound introduction. In these studies, we used white outbred mice weighing 18–22 g, which were on the standard diet for at least 7 days. All experiments were performed with ten mice per group.

For the prophylactic scheme the mice were intranasally (i.n.) inoculated with 0.2 ml of the tested compounds at $10 \times ID_{50}$ (50 %-mouse infectious dose; sub-lethal) 24 h before infection with the influenza virus. For the therapeutic scheme this procedure was carried out 24 h after infection of mice with the influenza virus. At the same time for both schemes, the control was a group of animals infected with the influenza virus without administration of the tested compounds. The influenza virus strain A/FM/1/47(H1N1) with infectious titer in the MDCK cell culture 4.5 lg ID_{50}, caused 100 % mortality of control group mice within 5 days. Observation of experimental animals was carried out for 7 days. It was also studied the effect of reference drugs in different regimens of administration to mice with experimental influenza pneumonia. As the reference drugs we used the known flu drugs Tamiflu® of pharmaceutical company F. Hoffman-La Roche, Switzerland (75 mg per capsule) and Rimantadine of pharmaceutical company "Darnitsa", Ukraine (50 mg tablets), which were administered at a dose of 10 mg/kg of animal weight.

The efficiency of protective action of the compounds was evaluated by the efficiency index (EI) of inhibition of lethality. EI of the tested substances was determined according to the formula [21]:

$$EI = \frac{PC - 1}{PC} \times 100 \text{ %},$$

where PC — protection coefficient defined as the ratio of the number of animals at the beginning to that at the end of experiment.

Results and Discussion

The anti-influenza activity of new synthesized adamantyl (alkyl, cycloalkyl) derivatives of aminopropanol-2 was studied against the influenza virus strain A/FM/1/47(H1N1). The antiviral activity of tested compounds 5, 6, 30, 33, 40, 47, 48, 51 was evaluated *in vitro* experiments by their chemotherapeutic index (CTI) determined as the ratio of the maximum tolerated dose (MTD) to the minimum active concentration (MAC). MTD of the tested compounds was determined by their cytotoxic effect on a monolayer of the MDCK cells culture. For this purpose, after 24-h cell growth the nutrient medium was replaced with the medium containing the tested compound in the concentration from 3.12 µg/ml to 100 µg/ml. In the control, no replacement was carried out. For the experiments at least ten wells with the cell culture were taken for each dilution of the compound. The level of CPE was determined
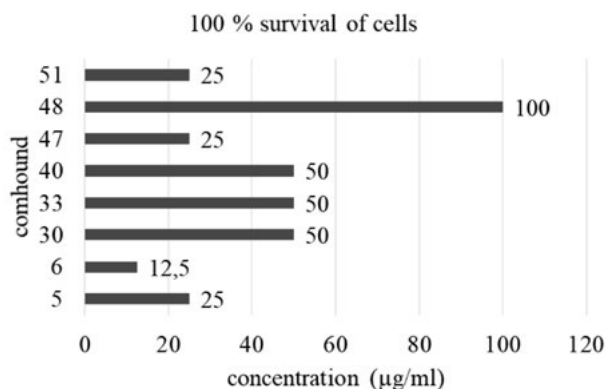
100 % survival of cells

Fig. 1. The MTD of test compounds adamantyl (alkyl, cycloalkyl) of aminopropanol-2 derivatives

by the change in cell morphology (degeneration or exfoliation of cells from the hole surface). MTD of the tested compound was taken as its highest concentration that did not cause degeneration of cells in all ten wells. The results of MTD determination for the studied compounds are presented in figure 1.

The MAC index of the tested substances was determined in vitro in a monolayer of the MDCK cells cultures by the level of reduction of infectious titer of the virus, in accordance with the General established procedure [16]. With this purpose the test-virus in a dose of 100 ID_{50} in 0.1 ml was introduced into the MDCK cells culture and incubated for 1 h at 37 °C. Next, the virus was removed by washing with medium RPMI-1640, and the support medium (RPMI-1640 + 2% fetal serum) with the tested compounds in concentration from 0.39 mcg/ml to 50 µg/ml was added. A reduction of infectious titer in the cell cultures treated with different concentrations of the tested compounds not less than 2 lg ID_{50} in the experiment compared with control allowed us to establish MAC of the tested compounds for the influenza virus (see figure 2).

According to the data in fig. 2 the compounds 47 and 48 have no influence on the reproduction of influenza virus in the studied concentrations (0.36 µg/ml to 50 µg/ml), they inhibit the development of virus-specific CPE in concentrations less than 2.0 lg ID_{50}. The compounds 5, 6, 30, 33, 40, 51 inhibited the influenza virus reproduction more than 2.0 lg ID_{50} in concentrations from of 1.56 µg/ml (compound 5) to 6.25 µg/ml (the compound 40), which gives grounds to consider them to possess the antiviral activity. The compounds 30 and 33 were the most active against the influenza virus, their MAC was the smallest and amounted to < 0.39 µg/ml.
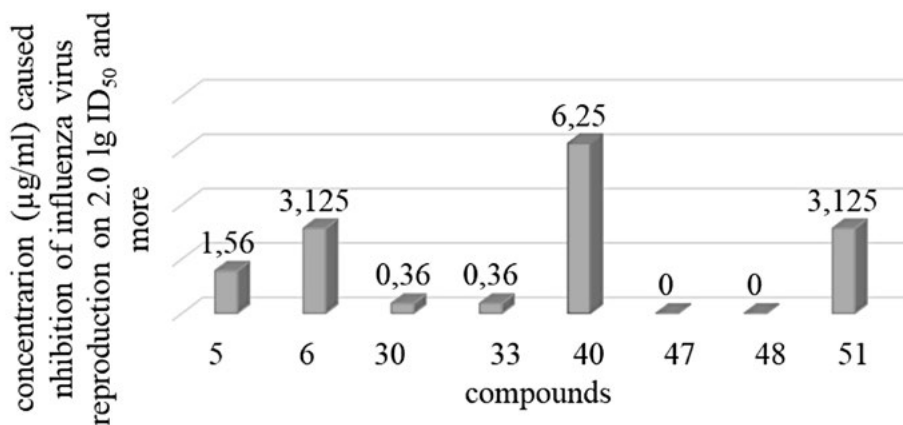

Fig. 2. The MAC of test compounds for influenza virus.

Table 1. **The value of CPE and MAC of anti-influenza drugs in relation to the influenza virus**

| Concentration of drugs µg/ml | anti-influenza drugs | | | |
| | Rimantadine | | Tamiflu | |
	CPE	Infectious titer of influenza virus (lg ID_{50})	CPE	Infectious titer of influenza virus (lg ID_{50})
500.0	0	NT	0	NT
250.0	0	NT	100	< 1.0
125.0	0	NT	100	< 1.0
62.0	100	4.0	100	< 1.0
31.0	100	4.0	100	< 1.0
15.5	100	4.0	100	< 1.0
7.75	100	4.0	100	4.0
Control of virus	100	4.0	100	4.0

* NT – not tested

MTD of anti- influenza drugs Rimantadine and Tamiflu was determined by CPE on the monolayer of the MDCK cell culture, and the value of their MAC was established by the degree of reduction of infectious titer of the influenza virus (Table 1). The data in the table 1 show that Rimantadine and Tamiflu were non-toxic to the MDCK cell cultures in a dose of 62.0 µg/ml and 250.0 µg/ml respectively, that corresponds to the value of MTD of these anti-influenza drugs.

The value of ratio of MTD to MAC of the studied compounds, and that of anti-influenza drugs Tamiflu and Rimantadine allowed us to determine their CTI. These data are shown in table 2.

Table 2. **MTD, MAC and CTI of the tested compounds, anti-influenza drugs Tamiflu and Rimantadine**

compounds	MTD, mcg/ml	MAC, mcg/ml	CTI
5	25.0	1.56	16
6	12.5	3.125	4
30	> 50.0	0.36	256
33	> 50.0	0.36	256
40	> 50.0	6.25	8
47	12.5	0	–
48	< 100	0	–
51	12.5	3.125	4
Rimantadine	6200	0.36	172.2
Tamiflu	25 000	0.02	12 500

Table 3. **The titer of HAI for compounds 5, 30, 33**

| Concentration of compounds µg/ml | The titer of HAI* tested compounds | | |
	5	30	33
50.0	–	–	–
25.0	–	++++(1/512)	–
12.5	–	++++(1/1024)	–
6.25	–	++++(1/1024)	–
3.1	–	++++(1/1024)	–
1.55	–	++++(1/1024)	–
0.72	++++(1/512)	++++(1/1024)	–
0.36	++++(1/1024)	++++(1/1024)	–
Control of virus	++++(1/1024)	++++(1/1024)	++++(1/512)

HAI * «-» – absence of hemagglutination ; «++++» – presence of hemagglutination,titer of HAI

Table 4. **Anti-influenza action of the compounds 5, 30, 33 and the anti-influenza drugs *in vivo* experiment.**

Substances	Concentration of substances mcg/ml	The number of mice		Protection coefficient	Efficiency index (EI), %
		the total number	of them died, (%)		
prophylactic scheme					
5	1.55	10	4 (40 %)	2.5	60
30	0.36	10	4 (40 %)	2.5	60
33	0.36	10	8 (80 %)	1.25	20
Rimantadine	0.5	10	5 (50 %)	2	50
Tamiflu	0.02	10	2 (20 %)	5	80
therapeutic scheme					
5	1.55	10	0	> 10	100
30	0.36	10	4 (40 %)	2.5	60
33	0.36	10	0	> 10	100
Rimantadine	0.5	10	6 (60 %)	1,66	39.7
Tamiflu	0.02	10	2 (20 %)	5	80
Control of virus	0	10	10 (100 %)	–	–

The values of MTD, MAC and CTI of the compounds 30 and 33 correspond to the substances highly active against the influenza virus. CTI of these compounds was equal to 256 and was higher than the same index of the anti-influenza drug Rimantadine, CTI of which was 172.2. The compound 5 is active against the influenza virus, its CTI equal to 16. The value of CTI of the compounds 6, 40 and 51 was also more than 3 (4, 8 and 4 respectively) (Table 2).

It is known that cells are sensitive to the influenza virus, bearing on their surface the receptors to viral glycoprotein of hemagglutinin (HA). The braking hemagglutination activity of the viruses in some cases is associated with a loss of the virus infectivity; therefore, it is appropriate to evaluate the influence of compounds on the hemagglutinin properties of viruses. We examined the influence of the most active compounds 5, 30 and 33 on the hemagglutinate activity of the influenza virus using hemagglutination inhibition test (HAI) (Table 3).

The titer of hemagglutination inhibition was the approved limiting dilution, which gives a complete delay of the hemagglutination reaction. The results of determination of the effect of various concentrations of the tested compounds on hemaglutinin properties of the influenza virus are shown in table 3. The data in the table 3 show that the compounds 30 and 33 with the same value of MAC (< 0.39 µg/ml) make different effects on the hemaglutination properties of the influenza virus. The compound 33 influences the virus hemagglutinin in much smaller concentrations than the compound 30. The HAI titers for the compound № 33 is 0,36 µg/ml and the HAI titer for compound № 30 is only 50 µg/ml.

The anti-influenza activity of the tested compounds 5, 30 and 33 was determined in the *in vivo* experiments on the model of mice

influenza pneumonia. The efficiency of the protective action of the tested compounds and anti-influenza drugs in the prophylactic and therapeutic schemes of their administration is shown in table 4.

Among the studied substances only the compounds 5 and 30 protect mice from the lethal influenza infection *in vivo* experiments in the prophylactic scheme of administration (Table 6). Their efficiency indices are equal (60 %) and higher than the Remantadine EI by 10 %. Unlike the substances 5 and 30 the compound 33 did not provide a significant protective effect in the prophylactic scheme of administration. EI of the compound 33 was only 20 %, which is much less than that of Tamiflu (80 %). The compounds 5 and 33 showed a much greater protective effect in the therapeutic scheme of administration than in the prophylactic scheme of *in vivo* experiment. EI of these compounds was 100 % in the therapeutic scheme of administration, compared

to the prophylactic scheme, where their EI values were 60 % and 20 % respectively. Comparison of the protective effects of newly synthesized compounds 5, 30, 33 and anti-influenza drugs Rimantadine and Tamiflu on the model of influenza pneumonia of mice in the prophylactic and therapeutic schemes of their administration is shown in figure 3.

The EI values of tested compounds 5, 30, and 33 were significantly different for different schemes of the *in vivo* experiment (Fig. 3). The tested compounds were more effective in therapeutic scheme of their introduction, when influenza drugs showed the same effect in both variants of the experiment. Complete protection against experimental influenza infection in mice was observed for compounds 5 and 33 in therapeutic scheme of their introduction. Their EI values were 100 %, that is more than twice higher than Rimantadine EI. The compound 30 showed identical efficiency in different schemes of the *in vivo* experiment, its
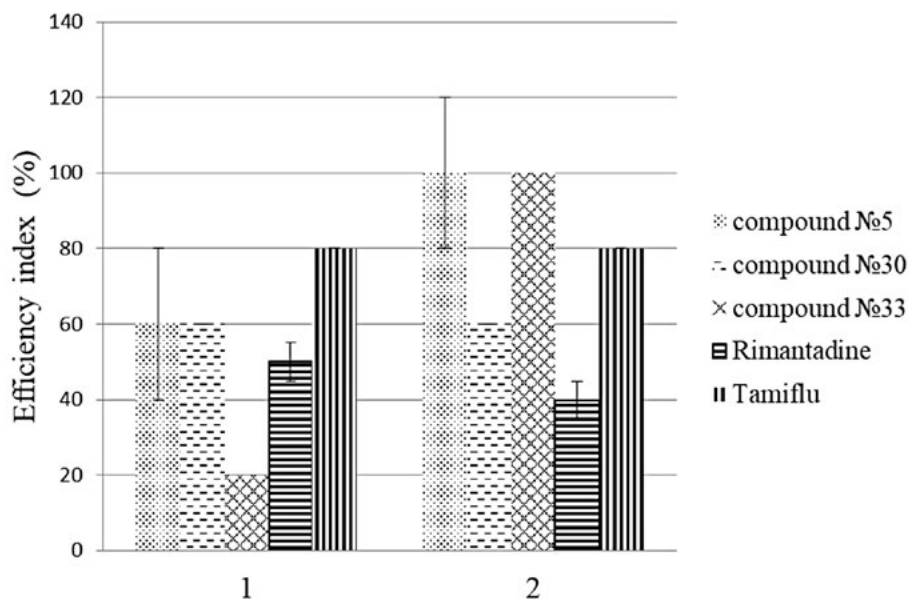

Fig. 3. Anti-influenza activity of the compounds 5, 30, 33 and anti-influenza drugs Rimantadine and Tamiflu on the model of influenza pneumonia of mice in the prophylactic (1) and therapeutic (2) schemes of their administration.

EI in both cases was 60 %. Noteworthy, for the compounds 30 and 33 an equally high CTI (256) was identified *in vitro* experiment, but these substances showed a different level of protective effect *in vivo* experiment.

Conclusions

Among eight tested adamantyl (alkyl, cycloalkyl) derivatives of aminopropanol-2 *in vitro* and *in vivo* experiments anti-influenza activity was found for the compounds 5, 30 and 33. CTI of the compounds 30 and 33 was equally high *in vitro* experiment on the MDCK cell culture, but these compounds showed different degree of protective effect *in vivo* experiment on the influenza pneumonia mice model, as well as different influence on hemagglutinin properties of the influenza virus. The compounds 5 and 33 were more effective in the therapeutic scheme of the *in vivo* experiment, whereas the anti-influenza drugs showed equal effect in both variants of the experiment (the therapeutic and prophylactic schemes of their administration). We suggest that these results may be explained by different mechanisms of action of the tested compounds on the flu virus.

The data obtained give ground to further research of the compounds 5, 30 and 33 for possible elaboration of new antimicrobial drugs on their basis.

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Антигрипозна дія адамантил (алкіл, циклоалкіл) похідних амінопропанолу-2 в дослідах *in vitro* та *in vivo*

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Мета. Визначити антигрипозну активність у 8 сполук адамантил (алкіл, циклоалкіл) похідних амінопропанолу-2 в дослідах *in vitro* та *in vivo*. **Методи.** Оцінку противірусної дії сполук проводили in vitro за зниженням інфекційного титру вірусу грипу на культурі клітин, та *in vivo* на моделі грипозної пневмонії у мишей. **Результати.** В досліді *in vitro* 3 сполуки серед 8 досліджених пригнічують репродукцію вірусу грипу штаму A/FM/1/47(H1N1) на 2 lg ID_{50} і більше в концентраціях від < 0,39 мкг/мл (сполуки №№ 30 і 33) до 1,56 мкг/мл (сполука № 5), їх хіміотерапевтичні індекси стосовно цього вірусу становлять 256 та 16, відповідно. В досліді *in vivo* при профілактичній схемі досліду сполуки №№ 5 і 30 мають індекс ефективності (ІЕ) 60 %, а при лікувальній схемі досліду ІЕ сполук №№ 5 та 33 більше ніж в два рази перевищує цей показник у ремантадину і становить 100 %. **Висновки.** Високі хіміотерапевтичні індекси та здатність пригнічувати репродукцію вірусу грипу на 2 lg ID_{50} і більше дозволяє віднести сполуки №№ 30, 33 і 5 до таких, що проявляють протигрипозну активність. Це також підтверджено в досліді *in vivo*.

К л ю ч о в і   с л о в а: вірус грипу, адамантил (алкіл, циклоалкіл) похідні амінопропанолу-2, антивірусна дія.

Антигриппозное действие адамантил (алкил, циклоалкил) производных аминопропанола-2 в экспериментах *in vitro* и *in vivo*

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Цель. Определить антигриппозную активность у 8 соединений адамантил (алкил, циклоалкил) производных аминопропанола-2 в экспериментах *in vitro* и *in vivo*. **Методы.** Противовирусное действие исследуемых соединений определяли *in vitro* по снижению инфекционного титра вируса гриппа в культуре клеток MDCK и *in vivo* на модели гриппозной пневмонии у мышей. **Результаты.** 3 соединения среди 8 исследованных подавляют репродукцию вируса гриппа штамм A/FM/1/47(H1N1) на 2 lg ID_{50} и больше в концентрациях от < 0,39 мкг/мл (соединения №№ 30 и 33) до 1,56 мкг/мл (соединение № 5), их химиотерапевтические индексы (ХТИ) в отношении этого вируса составляют 256 и 16, соответственно. В эксперименте *in vivo* при профилактической схеме опыта соединения №№ 5 и 30 имеют индекс эффективности (ИЭ) 60 %. ИЭ соединений №№ 5 и 33 при лечебной схеме опыта больше чем в два раза превышает этот показатель у Ремантадина и составляет 100 %. **Выводы.** Высокие ХТИ и способность подавлять репродукцию вируса гриппа на 2 lg ID_{50} и более позволяют отнести соединения №№ 30, 33 и 5 к таким, что проявляют противогриппозную активность. Это также подтверждено в экспериментах *in vivo*.

К л ю ч е в ы е   с л о в а: вирус гриппа, адамантил (алкил, циклоалкил) производные аминопропанола-2, антивирусное действие.