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CYTOTOXIC AND ANTIVIRAL PROPERTIES OF 4,4'-BIS-AMINOALKOXYBIPHENYLS

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Summary

Despite the high efficiency of the etiotropic antiviral drugs and antiviral vaccines they can lead to the selection of resistant mutant variants, initiate of evolutionary "arms race" [1] and pose a threat of the superinfections and epidemics. In this regard, the creation of means that enhance non-specific antiviral resistance is actual task and will be such in the future. The development of such agents – the functional analogues of amixyne (tilorone) – inducers of IFN type I is conducted in the past decades in O.V. Bogatsky Physico-Chemical Institute of the National Academy of Sciences of Ukraine. As an inactive model compound 4,4'-bis-[2-(diethylamino)ethoxy]bi-phenyl was synthesized in 2005 and unexpectedly appeared as an active antiviral and interferon inductor [2]. In this regard, analogues of this compound is of considerable interest from the point of view of the structure-properties relationships. These substances (R-A-4DP4-A-R, R – amino group of different structure; A = -$(CH_2)_n$O-, n = 2 - 6; 4BP4 - 4,4'-biphenyl) were synthesized via halogenoalkylation of 4,4-dioxybiphenyl with dihalogenoalkanes following with amino dehalogenation.

Keywords: Cytotoxic; antiviral properties; aminoalkoksybiphenyls.

The aim: investigation of the aminoalkoxybiphenyls cytotoxic and antiviral properties

Materials and methods

Biphenyl derivatives (table 1) were used as test compounds. These compounds were synthesized in the Medical Chemistry department of O.V. Bogatsky Physico-Chemical Institute of the National Academy of Sciences of Ukraine. 1 Mg of corresponding compound was dissolved in 1 cm^3 bidistilled sterile water.

Aminoethoxy biphenyls properties were studied in continuous cell lines L929 - subcutaneous connective tissue cells mouse C3H / An, subline "a".

Vesicular stomatitis virus (VSV), Indiana strain was used as a test-virus.

Culturing the cells

Cells L929 in the logarithmic growth phase were used for experiments. EMEM medium containing 10 % fetal calf serum (FCS) or bovine serum (SB) and 1% EMEM was used for culturing. The procedure for the cells replanting-removing was carried out using 0.25 % trypsin and 0.02 % Versen solution. Cells were cultivated (recultivation – every 2 – 3 days) in monolayer culture using glass bottles and 199 medium supplemented with 10% fetal calf serum (FCS), 25 mM HEPES (pH 7,4) and 1.0 mg/ml kanamycin at 37 °C under constant of carbon dioxide (5%) as nutrient medium.

Cells were removed from the surface of bottles with a Versen solution, resuspended in nutrient medium and adjusted their concentration in suspension to 5×10^5 cells / cm^3. Formation of the cells monolayer was performed adding of 0.1 cm^3 portion of specified cell suspension into wells of 96-well flat-bottomed plates («Sarstedt», Germany) and incubation with a constant level of CO_2 (5 %) at 37 °C for 24 hours. Toxic and antiviral effects of compounds as well as the cells cytopathic action of virus were investigated in the supportive environment conditions (media that contained 2 % ECT).

The method of living cells counting

In order to establish the properties of the test substances, counting the living cells number was performed after staining with crystal violet. In order to establish the properties of the test substances, counting the number of living cells was performed after staining with crystal violet. Supernatant was removed from wells, and 0.4 % solution of the dye Crystal Violet («Sigma», USA) in 2 % ethanol have added to cells, incubated for 30 minutes at room temperature following dye removing and cell monolayer washing with water. Absorbance of the dyed cells was measured using the spectrophotometer Multiskan Ascent («Thermo Labsystems», Finland) at a wavelength of 540 nm [3]. Calculation of living cells was performed as a percentage of the experimental wells' absorbance relatively to the last of the wells with intact cells. Both absorbances were taken with subtracting the absorbance of the wells in which the cells were absent.

Studying the toxicity of substances *in vitro*

Lines of murine fibroblast L929 cells were obtained from the Research Institute of

Veterinary Medicine, Ukrainian Academy of Agriculture, Kiev, Ukraine. Solutions of the studied compounds (10 mg / cm^3) were placed into 96-well microtiter plates (FaCCon) with the formed monolayer cells followed with a serial double dilution. Cells were grown for 24 hours at 37 °C with a constant level of CO_2 (5 %). For each concentration of substances' solutions 3 wells with cells were used.

The toxicity of the compound was determined as the maximum tolerated concentration (MTC) by cells and as compound concentration that resulted in the 50 % cells death (LC_{50}). Cells not treated with studied compounds were used as control. MTC values were used as starting concentration in determining of the compounds antiviral activity.

Percentage of living cells was determined as the ratio of the mean optical density values of three wells with a certain concentration of the substance to the same mean for intact control. All data pairs (the compound concentration's logarithm versus the survival of cells) were fitted with sigmoidal curve "dose-effect" at confidential probability $\alpha = 0.99$ (Equation 1):

$$S,\% = S_{min} + \frac{S_{max} - S_{min}}{1 + 10^{(lgC_{50} - lgC)p}}, \qquad (1)$$

where: $S,\%$ is a percentage of the living cells; S_{min} and S_{max} – approximation parameters corresponding to the smallest and largest cell survival, respectively (mostly insignificant different from 0 and 100%); lgC_{50} and lgC – logarithm of the compound concentration caused 50 % of cell death and current one, respectively; p – approximation parameter that characterizes the steepness of the dose-effect curve.

All dependencies of the living cells percentage from the test compounds concentration were treated similarly. Values of $-lgC_{50}$ were used for the structure-properties analysis.

Antiviral activity of the compounds *in vitro*.

Vesicular stomatitis virus (VSV) was grown in L929 cells to titers of about 106 ID50/0.1 mL. Assay of antiviral effects of compounds was based on evaluation of virus cell pathogen effects for VSV. The tested compounds properly diluted in appropriate media were added to cell monolayer grown in 96-well microtiter plates (FaCCon) at 37 °C in 5 % CO_2 (0.2 mL per well) either 24 h before ("preventive effect") or immediately after virus infection ("therapeutic effect"). Concentrations range 0.1 – 100 mM of compounds was used. Triplicate wells were employed as a rule. The multiplicity of infection was 0.1 ID50/cell for VSV. Virus yields (virus cytodestructive action) were estimated 24 h post infection. EC50, concentration (M) which led to 50 % cytopathic action of VVS inhibition, was determined using regression analysis method *(Microsoft Excel).*

Statistical evaluation of results was made by Student's t-test under $P < 0.05$.

Effective concentrations (ED_{50} and ED_{100}) were used to assess the antiviral activity of the studied compounds. The minimum concentration of the test substance, providing 50 % and 100 % protection of the cell monolayer from virus-induced cytopathic effect was taken as the ED100 and ED50, correspondingly.

In therapeutic schedule compounds were added to the culture medium in the plate wells at concentrations that do not exceed MTC, followed by two-fold serial dilution. The plates were incubated in an incubator at 37 °C during 24 h for the complete destruction of cells in control. Cells infected by the same test-virus in the absence of compounds were used as a positive control.

Results and Discussion

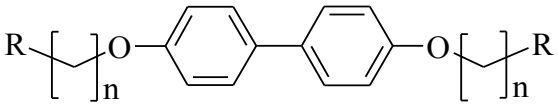
Structures of the biphenyl derivatives **1** – **30** used as test compounds are presented in table 1.

Cytotoxicity of compounds (tab. 2) **1** – **30** is varied in a wide concentration range. Thus LC50 value of compound **29** 2 μM (about 1 μg / cm^3), while the same for compound **3** reaches 8 mM (about 3.2 mg / cm^3).

The least toxic compound **3** contains morpholine as a terminal amino group and two methylene units in the side chain. The most toxic one is **29**, which is tetra acidic base. Tetra acidic compounds **5, 6, 11, 12, 17, 18, 23, 24, 29, 30** are insignificantly (at $P < 0.05$) more toxic then diacidic ones. Compounds cytotoxicity increases slowly while hydrocarbon chain extends from 2 to 4 methylene units, further lenthening (up to 5 and 6 methylene units) results in the sharp increases of toxicity. Toxicity of compounds with 5 and 6 methylene units in side chain are practically equal.

Table 1

The structure and numbering aminoalkoxybiphenyls general formula



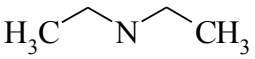
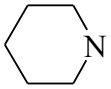
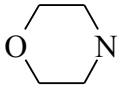
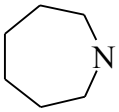
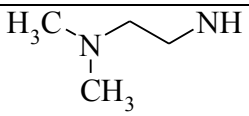
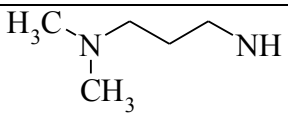
R	*n*				
	2	*3*	*4*	*5*	*6*
	**1**	**7**	**13**	**19**	**25**
	**2**	**8**	**14**	**20**	**26**
	**3**	**9**	**15**	**21**	**27**
	**4**	**10**	**16**	**22**	**28**
	**5**	**11**	**17**	**23**	**29**
	**6**	**12**	**18**	**24**	**30**

Table 2

Cytotoxicity of the aminoalkoxybiphenyls

Compounds	-*lg*LC_{50}, M	Compounds	-*lg*LC_{50}, M	Compounds	-*lg*LC_{50}, M
1	2.5	**7**	3.5	**13**	4.0
19	5.2	**25**	5.3	**2**	3.0
8	3.5	**14**	4.6	**20**	5.4
26	5.3	**3**	2.1	**9**	2.6
15	2.8	**21**	5.2	**27**	5.6
4	3.5	**10**	4.0	**16**	4.4
22	5.2	**28**	5.5	**5**	3.0
11	4.0	**17**	5.0	**23**	5.5
29	5.7	**6**	2.9	**12**	4.3
18	4.5	**24**	5.4	**30**	5.5

Elongation of the polymethylene chain causes the increasing of cytotoxicity by three orders of magnitude. Cytotoxicity of compounds with equal length chains of but with different terminal amino groups appeared in the same order of magnitude. Thus, elongation chains are a key factor in the growth of toxicity. The same tendency was observed in the dependence of these compounds' hemolytic properties from their structure [4].

Aminoalkoxybiphenyls **1** – **30** show a marked antiviral activity in the model the VSV infection on L929 cells in the therapeutic scheme. In the preventive scheme effect is much lower and not significantly different from "untreated" control cells. The dependence of the antiviral activity from structure is opposite to that one for cytotoxicity.

Thus, antiviral properties are the characteristic property of 4,4'-bis-

aminoalkoxybiphenyls as a class of compounds, which is in agreement with the previously shown the presence of these properties of two compounds of this class [5].

Unlike cytotoxicity antiviral activity demonstrates tends to decrease almost by one order if chain is elongated. The significance of the structural factors' impact for antiviral activity is similar to their influence on the cytotoxicity, although the influence of the chain length is less pronounced than in the case of cytotoxicity.

Table 4

Antiviral activity of the aminoalkoxybiphenyls

Compounds	-*lg*LC_{50}, M	Compounds	-*lg*LC_{50}, M	Compounds	-*lg*LC_{50}, M
1	6.3	**7**	6.0	**13**	5.8
19	5.6	**25**	5.5	**2**	6.2
8	6.1	**14**	6.0	**20**	5.8
26	5.6	**4**	6.3	**10**	6.2
16	5.9	**22**	5.7	**28**	5.3

To assess the significance of structure factors (chain length and amino group structure) we have fulfilled two-factor ANOVA whole array of cytotoxicity and antiviral activity results. In case of cytotoxicity both factors are significant at a high level of confidential probability ($P = 0.005$ and $P = 2.01 \times 10^{-10}$, respectively). The side chain length provides almost 83% of cytotoxicity changes while structure of the terminal amino group – just over 9%. In case of antiviral activity influence of amino group structure appeared as insignificant ($P = 0.287$), while the side chain length is significant at a high level of confidence probability ($P = 5.5 \times 10^{-5}$). Length of the side chain input provides almost 92 % change activity in while structure terminal amino group – just over 2% (Fig. 1).

Thus, compounds with shorter chain should be considered as promising in both terms of cytotoxicity and antiviral activity.

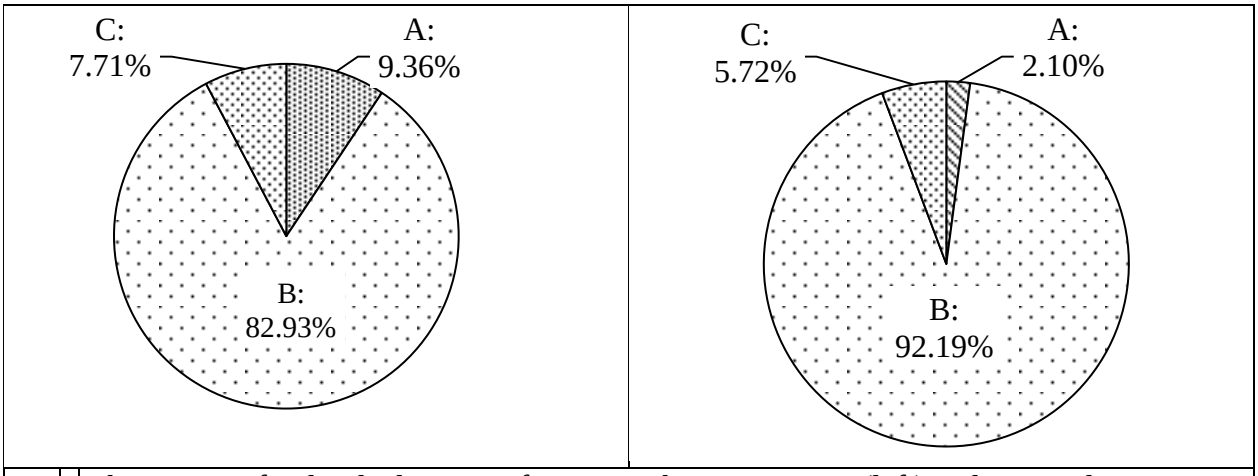


Fig. 1 The impact of individual structur factors on the cytotoxicity (left) and antiviral activity (right) of compounds. A – the structure of the terminal amino group; B – the length of the side chain; C – uncertain factors

Conclusions

1. In the model of the VSV infection on L929 cells aminoalkoxybiphenyls show a marked antiviral activity under simultaneous with virus introduction of the investigated compounds.
2. Aminoalkoxy biphenyls cytotoxicity increases with the increasing of the methylene units number in the side chain while dependence of antiviral activity from structure is opposite.
3. Replacing the terminal amino group does not introduce any significant effect on the

antiviral activity of compounds contrary to cytotoxicity.

4. The similarity cytotoxic and hemolytic activity indicates that plasma membrane damage can be a major implementation mechanism of cytotoxicity.

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