SYNTHESIS AND BIOLOGICAL ACTIVITY OF WATER-SOLUBLE POLYMER COMPLEXES OF ARBIDOL

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We have synthesized water-soluble complexes between the antiviral drug arbidol and polymer compounds with molecular masses of 19 – 31 kDa representing copolymers of acrylamide (AA) and 2-acrylamido-2-methylpropanesulfonic acid (AAMPS). The complexes are less toxic than arbidol and retain the high level of antiviral activity of this drug. The content of arbidol in the obtained complexes is within 26.4 – 32.1 mass%. The antiviral activity of the synthesized polymeric complexes against all studied viruses, including human epidemic influenza virus A (H3N2), bird highly pathogenic influenza virus A (H5N1), herpes type 1 virus (HSV-1), and adenovirus type III (AV-III) is comparable to the antiviral effect of nonmodified arbidol. The *in vitro* toxicity of the obtained complexes is about one order of magnitude lower than that of nonmodified arbidol; the pharmacological index, four times that of the initial low-molecular-weight drug. The synthesized water-soluble polymer complexes of arbidol can be useful in pharmacology since they can serve as the basis for new effective and safe parent antiviral substances and related formulations.

Key words: water-soluble polymer complexes of arbidol, antiviral activity, use in pharmacology.

The current domestic antiviral agent arbidol (6-bromo-4-dimethylaminomethyl-5-hydroxy-1-methyl-2-phenylthiom ethylindole-3-carboxylic acid ethyl ester hydrochloride monohydrate) exhibits a broad spectrum of activity against flu A and B viruses and other acute respiratory viral infections (SARS). Arbidol is also an immunomodulator, interferon inductor, and antioxidant [1]. The virus-inhibiting activity of arbidol is due to its ability to inhibit fusion of the virus lipid shell with membranes of endosomes located within the cells [2]. At present arbidol is widely distributed on the domestic pharmaceutical market and is one of the main etiotropic antiflu preparations.

However, the fact that the preparation is practically insoluble in water is an important drawback. This decreases its bioavailability and limits the ability to create new drug forms based on it (solutions, aerosols, hydrophilic lotions). Furthermore, arbidol is effective as a drug only in the early stages of the flu, not more than two days after the start of the disease [3].

Arbidol preparations have low toxicity if used internally [1]. However, parenteral administration in mice and rats gave DL_{50} values of 109 and 140 mg/kg, respectively. This indicated its preparations were moderately toxic [4]. Tests in cell cultures estimated its IC_{50} (average inhibiting concentration reducing cell viability by 50%) as 40 – 60 μg/mL [2]. Furthermore, according to our data, storage of the preparation for about six months increases substantially its cytotoxicity (IC_{50} decreases to 20 μg/mL in MDCK cell culture). Also, the pharmacological index (selectivity index) is only 3.75, which is an unacceptable index for such a widely used preparation (as a rule, the selectivity index should be at least >10) [5].

Complexation with water-soluble nontoxic ionogenic polymer-carriers is one effective and technical method for reducing the toxicity of drugs while retaining specific biological activity [6]. Polymeric preparations of remantadinum (its complex with sodium alginate algirem and complex with vinylamidosuccinic acid polirem) exhibit a broad spectrum of antiviral activity compared with remantadinum. A contributing factor is the prolonged effect of polymeric remantadinum derivatives that are capable of lengthy circulation of the antiviral component at effective therapeutic doses [7].

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Our goal was to prepare water-soluble arbidol derivatives with copolymers of acrylamide-2-acrylamido-2-methylpropanesulfonic acid (AAMPS) with reduced toxicity and retention of the high level of antiviral activity.

Copolymers of AAMPS were chosen as modifiers of arbidol properties because these copolymers, like all acrylamide (AA) polymers, have highly hydrophilic polymer chains that dissolve well in water; contain strongly acidic sulfonic acids and readily form salt bonds to primary, secondary, and tertiary amines in aqueous solutions; and are nontoxic *in vitro* according to our previous results for copolymers of AA with acrylic acid [8]. This suggested that the resulting copolymers containing a relatively small number of sulfonic acids (<23 mol%) would also be nontoxic.

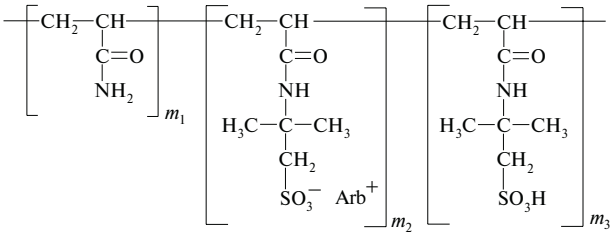
EXPERIMENTAL CHEMICAL PART

Acrylamide (Serva, Germany) was recrystallized from benzene. 2-Acrylamido-2-methylpropanesulfonic acid (Yaroslav Polytechnical Institute) was recrystallized from acetic acid with added acetic anhydride by the published method [6].

Polymer-carriers were AA copolymers with AAMPS and were prepared by heterophase radical copolymerization of the comonomers in isopropanol induced by azoisobutyronitrile using the ampul method [6]. The composition of AA–AAMPS copolymers was established using elemental analysis for S content. Molecular weights of AA–AAMPS copolymers were determined by viscosimetry using the Mark–Kuhn–Houwink equation that is known for polyacrylamide [9].

Complexation of AA–AAMPS copolymers with arbidol (Masterlek, Moscow, capsule form) was carried out in water at room temperature with copolymer:arbidol mass ratio 2.3 – 2.5:1. Copolymers were dissolved in distilled water at 3.0 – 4.0 mg/mL, stirred, and treated with dry arbidol (26 – 30 mg). The mixture was stirred for 40 – 60 min, during which all arbidol dissolved completely. The mixture was filtered. Polymeric complexes were isolated by lyophilization. The arbidol content in the resulting polymeric complexes was determined by UV spectroscopy using the strong arbidol absorption band with a maximum at 320 nm ($\varepsilon = 13{,}700\ mol^{-1} \cdot cm^{-1}$). UV spectra were recorded in DMF + H_2O (1:9, v/v).

The resulting complexes of copolymers with arbidol had the following structure and composition:



Arb = arbidol,
$m_1 = 100 - (m_2 + m_3)$, mol%;
$m_2 = (7{,}6 – 9{,}8)$ mol%;
$m_3 = (11{,}5 – 13{,}6)$ mol%;
MW = 19000 – 31000 Da;
arbidol content 26.4 – 32.1 mass%

EXPERIMENTAL BIOLOGICAL PART

Cytotoxicity of obtained compounds *in vitro*. The toxicity of arbidol, polymer-carriers, and polymeric complexes of arbidol were determined by incubation of MDCK cell cultures with successive dilutions of the preparations in Igla-MEM serum-free medium for 72 h. Toxicity *in vitro* was estimated using reduction by cells in culture of tetrazolium dyes resazurin (Sigma, USA) (fluorimetric method) or MTT (ICN Pharmaceuticals, USA) (photometric method) [10, 11].

The toxicity criterion in both methods was IC_{50}, the cytotoxic dose causing a reduction of the corresponding parameter by 50% of the control (intact cells). It was calculated using dose—effect linear regression equations.

Antiviral activity *in vitro*. Antiviral activity of the complexes was determined against the standard strain of human flu virus A/Victoria/35/72 (H3N2) and a highly pathogenic bird flu strain isolated and characterized at the RII A/duck/Kurgan/8/05 (H5N1) [12].

The presence of virus in the incubation medium was determined using a hemagglutination micromethod. Virus titre was expressed in logarithm per 100 μL (log TID_{50}), where TID_{50} is the 50% tissue inhibiting dose. Antiviral activity was estimated from the reduction of virus titre in planchet test wells compared with controls ($\Delta\log TID_{50}$). The average virus-inhibiting concentration (VIC_{50}) was calculated from the cytopathic reaction of cells treated with virus of a certain titre that was estimated by the aforementioned MTT method. The degree of cell viability inhibition in culture correlated with the development of viral infection *in vitro*.

Antiviral activity was also estimated against herpes simplex virus 1 HSV1/248/88 and adenovirus III Ad/3/et/4120 in A-549 cell culture, which is sensitive to these viruses. Samples were introduced according to a therapeutic-prophylactic scheme, i.e., 30 min before addition of virus suspension to the culture. The cytopathic reaction was calculated after 48 and 72 h from the degree of monolayer degradation (microscopic study of the culture) and by the MTT method. In the latter instance the aforementioned regression method was used to calculate VIC_{50}.

RESULTS AND DISCUSSION

Tables 1 – 3 and Figs. 1 and 2 give the composition and biological characteristics of the products. Table 1 shows that the antiviral activity of the arbidol complex was comparable with that of nonmodified preparation against model strain flu A virus (H3N2). Its toxicity *in vitro* was about 10 times less than that of starting arbidol (corresponding IC_{50} was higher by an order of magnitude). The antiviral activities of various

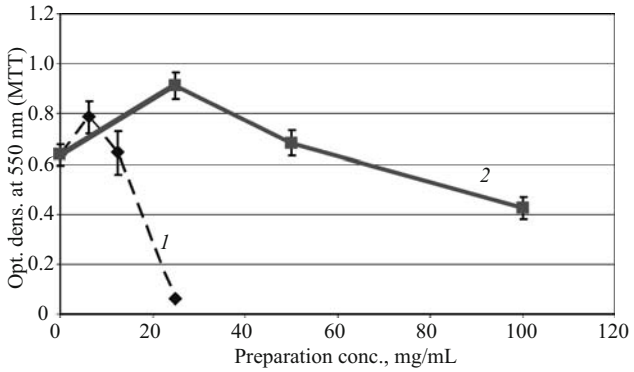


Fig. 1. Cytotoxicity of arbidol (1) and Arb–AA–AAMPS complex (2) in MDCK cell culture. IC_{50} of arbidol 19.5 μg/mL; of Arb–AA–AAMPS, 190 μg/mL. Optical density at 550 nm (MTT) along the ordinate.

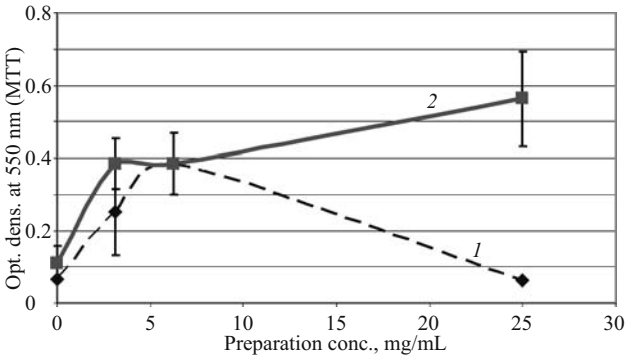


Fig. 2. Antiviral activity of arbidol (1) and Arb–AA–AAMPS complex (2) against highly pathogenic bird flu A A/duck/Kurgan/8/05. VIC_{50} of arbidol at 10 TID_{50}, 5.2 μg/mL; VIC_{50} of complex under the same conditions, 12.5 μg/mL. Optical density at 550 nm (MTT) along the ordinate.

variants of the complex (Nos. 1 – 4, Table 1) were about equal. The corresponding parameter ($\Delta\log TID_{50}$) exceeded 2.0, which indicated that the antiviral activity was rather high. The antiviral activities of complex variants Nos. 1 – 4 were not statistically different from each other and from the activity of nonmodified arbidol (nonparametric Mann–Whitney criterion). The toxicities *in vitro* of all complexes studied in examples 1 – 4 were approximately the same. A slight oscillation of cytotoxicity (statistically insignificant) was observed depending on the sulfonic-acid content (Table 1).

Table 2 shows that arbidol in this system exhibited pronounced antiviral dose-dependent activity ($\Delta\log TID_{50} > 2.0$) against not only model human flu A virus (H3N2) but also the highly pathogenic bird flu A strain (H5N1). The activity of the complex against the latter was much higher than that of nonmodified arbidol.

Figures 1 and 2 show more detailed data for the activity of polymer-modified arbidol against H5N1. For 10 TID_{50}, VIC_{50} of arbidol was 5.2 μg/mL. The average toxic *in vitro* arbidol concentration against MDCK cells under these experimental conditions was 19.5 μg/mL. Thus, the pharmacological index (selectivity index of the preparation) for 10 TID_{50} was 3.75, which was clearly unsatisfactory for such a widely used preparation.

TABLE 1. Chemical and Biological Properties of Arbidol Polymer Complexes

No. of arbidol–polymer complex	AA–AAMPS copolymer		Polymer/Arb mass ratio in synthesis	Arbidol polymer complex					
	Sulfonic content in starting polymer, *n*, mol%	MW, Da		yield, %	m_2, mol%	m_3, mol%	Arb content, mass%	Toxicity, IC_{50}, μg/mL*	Antiviral activity against flu virus A (H3N2), $\Delta\log TID_{50}$**
1	19.1	19 000	2.5:1	78.8	7.3	11.8	28.1	470 ± 52	2.25
2	22.0	31 000	2.5:1	83.2	7.8	14.2	28.6	461 ± 45	2.0
3	22.8	28 000	2.5:1	83.6	7.0	15.8	26.4	435 ± 31	2.2
4	22.8	28 000	2.3:1	87.9	9.3	13.5	32.1	475 ± 52	2.33
5 (control, low arbidol content)	22.8	28 000	3.0 : 1	84.4	6.5	16.3	24.8	480 ± 72	1.0
6 (control, insoluble)	19.1	19 000	1.95:1	Arbidol polymer complex soluble in water not obtained			34.0***	–	–
Control for polymer-carrier (from Nos. 3 – 5)	22.8	28 000	–	–			–	17800 ± 3.410	0.25
Control, nonmodified arbidol	–	–	–	–			100	48.7 ± 8.5	2.62

* Average toxic concentration in MDCK cell culture.
** Decrease in flu virus hemagglutinizing activity expressed in logarithm of greatest virus dilution causing hemagglutination.
*** Calculated for loading at which a water-soluble arbidol polymer complex would be obtained under the given conditions.

TABLE 2. Antiviral Effect of Arbidol and Its Complex with AA—AAMPS* (No. 1 from Table 1) on Reproduction of Flu Virus A (H3N2) and Bird Flu Virus A (H5N1) in MDCK Cell Culture

Parameter	A/Victoria/35/72 (H3N2)				A/duck/Kurgan/8/05 (H5N1)		
	control	Arbidol, 10 μg/mL	Arb–AA–AAMPS, 30 μg/mL	Arb–AA–AAMPS, 60 μg/mL	control	Arbidol, 10 μg/mL	Arb–AA–AAMPS, 33 μg/mL
Hemagglutinizing activity (log TID_{50})**	5.5	2.88	3.25	2.25	5.5	3.75	3.25
Antiviral effect (Δlog TID_{50})	–	2.62	2.25	3.25	–	1.75	2.5

Preparations placed in culture medium 30 min before virus infection. Incubation of cells with virus in supporting medium for 48 h. Results calculated by HAR. Averages of four independent tests given.

* Weight concentrations of modified arbidol selected considering arbidol content in polymer complex (~30 mass%).

** Hemagglutinizing activity given in logarithm of greatest virus dilution causing hemagglutination reaction of human blood type O (I) erythrocytes (log of average tissue infection dose, TID_{50}). Antiviral effect given in difference of parameter between test and control samples (intact virus).

TABLE 3. Antiviral Effect of Arbidol and its Complex with AA–AAMPS (No. 1 from Table 1) Against Herpes Simplex Virus HSV1/248/88 and Adenovirus Ad/3/et/4/20 in A-549 Cell Culture

Parameter	Control	Arbidol, 10 μg/mL	Arb–AA–AAMPS, 30* μg/mL	Arb–AA–AAPS, 60 μg/mL
	Herpes virus/Adenovirus			
CPA, (log TID_{50}), microscopic morphology evaluation	3.67/3.0	1.83/2.25	2.5/2.13	2.13/2.25
Antiviral effect from microscopic evaluation (Δlog TID_{50})	–	1.84/0.75	1.17/0.87	1.54/0.75
CPA, (log TID_{50}), MTT reduction	2.6/2.04	0.55/1.3	0.95/1.5	0.4/1.9
Antiviral effect from MTT reduction (Δlog TID_{50})	–	2.1/0.74	1.65/0.54	2.2/0.14

Preparations added 30 min before virus infection. Results calculated after 72 h. Cytopathogenic activity (CPA) evaluated from cell morphology changes (light optical microscope) and from MTT dye reduction. Averages of 3 – 4 independent tests.

* See explanation in Table 2.

However, the VIC_{50} value was 12.5 μg/mL; the toxicity IC_{50}, 190 μg/mL for polymer-modified arbidol at an equimolar concentration (considering the approximate arbidol mass content in it) with 10 TID_{50}. Thus, the selectivity index for this complex was 15.2 under the same conditions, which is four times better than that of nonmodified arbidol.

Hence it can be concluded that polymer-modified arbidol not only exhibited high antiviral activity against highly pathogenic flu A virus strain (H5N1), which was isolated in Russia, but also was significantly less toxic and had a much higher pharmacological index than nonmodified arbidol.

Table 3 shows the antiviral effect of arbidol and its complex with AA–AAMPS (variant No. 1 from Table 1) against HSV1/248/88 and Ad/3/et/4/20. It was found that arbidol had a significant antiviral effect and that the dose-dependent effect of the tested complex was similar in magnitude against herpes simplex virus type 1. The antiviral activity was comparable to that against human flu virus. With respect to the antiviral activity of arbidol and its polymer-modified derivative against adenovirus, only a weak antiviral effect was observed. A concentration dependence of the latter was not observed.

Thus, water-soluble polymer complexes of arbitol with relatively low-molecular-weight acrylamide copolymers with 2-acrylamido-2-methylpropanesulfonic acid that exhibit substantial antiviral activity comparable to that of nonmodified arbidol were obtained. However, the *in vitro* toxicity of the synthesized complexes was about an order of magnitude less and the pharmacological index (calculated against H5N1) was four times greater than those of the nonmodified preparation under the same conditions.

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