I. A. Leneva, I. T. Fedyakina, T. A. Guskova, R. G. Glushkov
The Shemyankin and Ovchinikov Institute of Bio-organic Chemistry, Moscow

The sensitivity of various influenza virus strains to arbidol. The influence of arbidol in combination with other antiviral drugs on reproduction of influenza virus A.

Aim. *To study the antiviral activity of arbidol in relation to various antigen subtypes of influenza virus; to study the effectiveness of arbidol when used in combination with adamantine-type antiviral drugs, ribavirin and ribamidil in inhibiting influenza virus reproduction in cell culture.*
Material and Methods. *The activity of the drugs against viral reproduction was assessed according to the expression of viral antigens in virus-infected cells, determined by enzyme immunoassay.*
Results. *Arbidol is not inferior to adamantine drugs, neuraminidase inhibitors, ribavirin and ribamidil in its inhibiting effect on influenza virus A and B. Arbidol inhibits reproduction of human IVA antigen strains H1N1, H2N2, H3N2 and rimantadine-sensitive as well as rimantidine-resistant strains of the influenza virus. Arbidol inhibits the reproduction of strains of avian influenza, pathogenic to humans, H5N1 and H9N2, and H6N1 and H9N2, which have genes in common with H5N1 and H9N2. Arbidol's inhibitory effect on viral reproduction in cell culture was enhanced when it was used in combination with amantidine, rimantadine, ribavirin and ribamidil.*
Conclusion. *Arbidol has broad-spectrum antiviral action, and inhibits reproduction of various antigenic subtypes and rimantadine-resistant human IVA, avian viruses H5N1 and H9N2, and influenza viruses B and C.*

Key words: arbidol, influenza viruses A and B, combined chemotherapy of influenza

At the present time, along with its campaign of vaccination as a main defense against influenza, the World Health Organization (WHO) is recommending administration of etiotropic drugs, that is, drugs which target a specific point in the viral reproductive cycle[1]. Included in the first generation of such drugs are the adamantadine types, amantadine and rimantadine. They block the ion channels in the transmembrane of the virus protein M2, and impede the proton transport which lowers the pH of the virion, a necessary condition for the release of virus ribonucleoproteins from protein M1 and the beginning of the virus genome transcription. Amantadine and rimantadine molecules, having a diameter corresponding to these ion channels, inhibit proton transport, raising the pH level in the endosome, hindering release from M1, and stopping the reproduction of the virus [2]. However, the adamantadine drugs are not effective against influenza B, have serious side effects, and also cause a rapid and widespread growth of resistant strains of the virus [3].

The second generation of drugs includes neuraminidase inhibitors, which are taken in the form of inhalers or aerosol sprays (zanamivir) and tablets or capsules (oseltamivir). These preparations inhibit the function of viral neuraminidase, which blocks the release of new viral particles from the cells and further spread of the virus within the organism [4]. Not only are these drugs very expensive, zanamivir can cause irritation of the nasal passages, and oseltamivir can cause nausea and vomiting [5]. From

the foregoing, it is clear that these existing remedies are lass than ideal; thus, there is a need for creation of a new drug which works through a different pathway. Lately, there has been widespread use in Russia of the drug arbidol, confirmed by the Russian Federation Pharmicological Committee for the prevention and treatment of influenza groups A and B in adults and children. The high therapeutic effectiveness of this new representative of the indole group is a result of its broad-spectrum biological action, and is based on its immunomodulator, interferon inductor, antioxidant and virus-specific qualities [6]. Clinical trials among more than 10,000 patients, and also 15 years of use in medical practice, have shown arbidol's safety, and its lack of side effects [7].

The present work gives the results of studies of the effectiveness of arbidol against various strains of the influenza virus which are pathogenic in humans, and also its effectiveness against viral reproduction when used in combination with other drugs.

Materials and Methods

Viruses and cells. In the experiments we used single-layer cultures of embryonic dog kidney cells (MDCK). The viruses studied were human influenza group A strains A/PR/8/34 (H1N1), A/WSN/33 (H1N1), A/Singapore/1/57 (H2N2), A/Japan/305/57 (H2N2), A/Krasnodar/101/59 (H2N2), A/Taiwan/79 (H3N2), A/Mississipi/85 (H3N2); influenza group B strains B/Lee/40, B/Singapore/222/79; and group C strains C/SSSR/ and C/Leningrad, received from the Institute of Virology named after D. I. Ivanovsky RAMN. The viruses A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) were isolated from humans during the Hong Kong influenza outbreak. Viruses A/guineafowl/Hong Kong/G1/97 (H9N2) and A/duck/Hong Kong/W312/97 (H6N1) were isolated from birds in Hong Kong. Viruses A/Hong Kong/196/57, A/Hong Kong/1074/99, A/guineafowl/Hong Kong/G1/97, and A/guineafowl/Hong Kong/G1/97 (P3) are the property of St. Jude Children's Research Hospital, Memphis, TN. All experiments were conducted in Biosafety Level 3 facilities.

The study of antiviral actions of drugs in MDCK cells using the enzyme immunoassay method. MDCK cells were placed in 96-well plates (from Costar) with an average density of 35,000 cells per well and cultured in Eagle medium with 5% fetal calf serum and 10 mM glutamine to produce one full layer. The drugs or drug combinations under study were added to the cells in two-round concentration in 100 mcl Eagle medium. We added 100 mcl of this same medium to the virus control, and 200 mcl to the cell control. In the experiments with human influenza virus strains, the virus compounds and divisions under study were done in Eagle medium with added TPCK trypsin (2.5 mcg/ml). To study the combined effect of preparations on viral reproduction, each plate included wells with control cells (cells not infected by the virus); wells with control virus (cells infected by the virus); wells infected by the virus in the presence of the two preparations under study; and "control wells," infected by the virus, with one or the other of the studied preparations individually added. For the study of the combined effectiveness of the drugs, we added 50 mcl of the drug solution to the cells in 4-stage concentrations. To the "control wells" we added 50 mcl of one drug in 4-stage concentrations, along with 50 mcl of medium with trypsin (2.5 mcg/ml).

After incubating the cells with the given preparations for 30 min. at 37 degrees Celsius, we added 100 mcl of allantoic virus in Eagle medium (multiplicity of infection

0.1-1 MOI; in studies on combined preparations, 1-5 MOI) to the wells, except for the cell control wells. Then the plates were incubated for 17 hours in a 5% CO_2 environment at 37 degrees; then the cells were fixed with 80% acetone in PBS for 20 min., and thoroughly dried, after which point we determined the expression of viral antigens by enzyme immunoassay, as earlier written [8]. The percent inhibition of the studied combinations was determined according to the formula: % inhibition = 100 – [OD_{490} (experiment) – OD_{490}(cell control)]/ [OD_{490}(virus control without the combination) – OD_{490}(cell control)]. The drug concentration which reduced the OD_{490} by 50% was taken as the minimal inhibitory concentration 50 (MIC_{50}).

Results and Discussion

It is well-known that in humans, influenza can display antigens of type A, B, or C. In the human population, the main subtypes of group A which circulate are H1N1, H2N2 and H3N2, which in turn undergo continual changes in their antigen structure [9]. For this reason, the most important characteristic of an anti-influenza preparation must be a broad-spectrum effect across strains. Clinical experiments showing the high prophylactic and therapeutic effects of arbidol have been conducted during influenza outbreaks caused by A/H2N2 and H3N1, B/369, and associated variants [6]. However, laboratory diagnostics and identification of the influenza strains causing illness in the specific people in the experiments were not completed. Thus, direct data of arbidol's sensitivity to various strains of influenza, which cause illness in humans, has been lacking.

Table 1: The influence of antiviral drugs on viral reproduction of various strains of human influenza virus A, B and C in MDCK cell culture

Virus	% inhibition of viral reproduction						
	arbidol	amanta-dine	riманта-dine	virazol	ribamidil	zanam-ivir	Carbox-ylate osel-tamivir
Influenza Virus A: H1N1							
A/PR/8/34	80	50	40	59	59	75	73
A/WSN	85	--	--	--	--	--	--
H2N2							
A/Singapore/1/57	80	79	81	60	60	81	79
A/Japan/305/57	87	84	85	--	--	--	--
H3N2							
A/Taiwan/79	79	85	80	--	--	85	77
A/Mississippi/85	83	82	87	66	66	84	80
Influenza Virus B:							
B/Lee/40	60	6	8	--	--	65	68
Influenza Virus C:							
C/SSSR	20	n/a	10	--	--	--	--
C/Leningrad	22	15	15	--	--	--	--

Note: Drug concentrations were the following: arbidol, virazol, ribamidil, zanamivir, oseltamivir – 10 mcg/ml; amantadine, rimantadine – 5 mcg/ml in experiments with group A and 20 mcg/ml in experiments with groups B and C. "—" indicates reactions not studied; "n/a" = not active.

In our studies comparing arbidol's effects to the effects of other anti-influenza drugs on strains of influenza virus A and B, arbidol inhibited viral reproduction of all the tested group A strains at approximately the same level, that is, 80%; arbidol did no worse than, and in fact, often surpassed the effects of other anti-influenza preparations (Table 1). In contrast to amantadine and rimantadine, which had practically no influence on the reproduction of viral group B, arbidol, like the neuraminidase inhibitors, decreased viral reproduction by 60%. Inhibition of virus group C was weaker, at 20%.

It is known that one defect of anti-influenza drugs is the rapid mutation of resistant strains to the drugs. This rapid mutation (within 1-2 trials) of drug-resistant strains has been shown in many experiments in animals and cell cultures; resistant strains in the human population can develop as soon as 2-4 days after treatment with these drugs. The circulation of these strains (moreover, with a relatively high level of virulence) in the human population has become a reality [2, 3]. Therefore, conquering these infections requires expedient use of drugs that work through a different mechanism. Our previous data points to the difference between the antiviral actions of arbidol and rimantadine [10]. Because of this, the study of arbidol's effect on strains of influenza which are resistant to rimantadine is of undoubted interest. Studies of arbidol's effects on the reproduction of strains sensitive to rimantadine and strains resistant to rimantadine have shown that arbidol inhibits the reproduction of strains both sensitive and resistant to rimantadine (Table 2).

Table 2: Influence of antiviral drugs on viral reproduction of rimantadine-sensitive and rimantadine-resistant strains of influenza A in MDCK cell culture

Viral avian illness – Weybridge strain (H7N7)	% inhibition OP 490	
	rimantadine (5 mcg/ml)	arbidol (10 mcg/ml)
Wild-type, rimantadine sensitive	56 +/- 4	60 +/- 3
Rimantadine-resistant	15 +/- 5	80 +/- 4

For most of the past century, it was widely accepted that only the antigens of influenza virus groups B and C and H1N1, H2N2 and H3N2 of group A could cause infections in humans. But in 1997, the avian virus H5N1 caused 18 cases of illness in Hong Kong, 6 of them resulting in death [11]. At the end of 2003 and beginning of 2004, a new outbreak of influenza caused by avian viruses appeared in various Asian countries, again with fatalities. The WHO recommended administration of anti-viral chemical preparations as one of the measures against the "bird flu." Virus A/Hong Kong/157/97 and A/guineafowl/Hong Kong/G1/97, causing infection in humans, and also the third avian virus A/duck/Hong Kong/W312/97 (H6N1) have six common genes which code internal proteins. Still another avian virus, A/chicken/Hong Kong/G9/97, has two genes (PB1 and PB2) in common with the abovementioned three viruses. These viruses continue to circulate among bird populations, and we cannot rule out the possibility of

their crossing the barrier between species and entering the human population. At present they are considered candidates for future influenza virus epidemics [12].

For this reason, we decided to study arbidol's effect on the reproduction of avian viruses A/Hong Kong/157/97 (H5N1), A/guineafowl/Hong Kong/G1/97 (H9N2), A/chicken/Hong Kong/G9/9797 (H9N2), and A/duck/Hong Kong/W312/97 (H6N1) in MDCK cell culture. Arbidol inhibited reproduction in all of the studied avian viruses, although they varied in their sensitivity to it. The MIC_{50} for both H9N2 viruses was 15 mcg/ml, at the same time that it was 30 and 25 mcg/ml, respectively, for A/Hong Kong/157/97 (H5N1) and A/duck/Hong Kong/W312/97 (H6N1). The MIC_{50} for virus A/Singapore/1/57, taken as a control, was 10 mcg/ml (Table 3).

Table 3: Antiviral activity of arbidol on avian influenza A viruses, having genes in common with H5N1 which code for internal proteins, in MDCK cell culture

Virus	Genes shared with A/Hong Kong/157/97 (H5N1)	MIC_{50}, mcg/ml
A/Hong Kong/157/97 (H5N1)		30 +/- 5
A/guineafowl/Hong Kong/G1/97 (H9N2)	PB1, PB2, PA, M, NS, NP	15 +/- 3
A/duck/Hong Kong/W312/97 (H6N1)	PB1, PB2, PA, M, NS, NP, NA	25 +/- 3
A/chicken/Hong Kong/G9/9797 (H9N2)	PB1, PB2	15 +/- 5
A/Singapore/1/57 (H2N2)		10 +/- 4

Thus, arbidol suppresses the reproduction of avian viruses H5N1 and H9N2, which cause infection in humans, and also represses reproduction of other avian viruses which have genes in common with them – although arbidol is less effective on these viruses than it is on H1N1, H2N2, and H3N2 antigen subgroups of the influenza virus circulating in the human population.

Combining one anti-influenza chemical preparation with another preparation is one way to increase the effectiveness of treatment. With this method, the best results are obtained by a combination of two drugs which work through different mechanisms, and operate independently on different stages of the interaction of the virus with cells. The study of arbidol's effects on different stages of viral reproduction showed that it inhibits the fusion of the viral lipid coat with the endosome membrane of the cell, preventing the entrance of the virus into the cell and the subsequent release of the viral genome within the cell and start of transcription [10, 13]. That process is induced by the conformational changes in the influenza virus surface protein hemagglutinin that take place during a lowered pH, allowing conditions facilitating membrane fusion. Experiments done with monoclonal antibodies on the conformational changes of hemagglutinin have shown that arbidol changes the conformation of hemagglutinin, preventing it from destabilizing to a more active form which would allow it to induce membrane fusion. These findings were confirmed through determination of nucleotide sequences from mutants resistant to arbidol, which were found to have mutations only at the gene which codes for hemagglutinin [14]. Taking into account our earlier data on the mechanism of action of arbidol, and the differences between this mechanism and the effective mechanism of

other anti-influenza drugs, we could study the combined effect of arbidol with other anti-viral preparations on viral reproduction in MDK cell cultures.

At present, anti-influenza chemotherapy widely incorporates amantadine and its relative, rimantadine. Aside from the fact that these preparations are ineffective against influenza group B, their usefulness is limited by the rapid growth of resistant strains to them, and by the side effects which high doses of these drugs produce [2, 3]. Often ribavirin (or its structural relative ribamidil) is used in the treatment of RSV. These preparations exist in the form of inhalants, or are administered intravenously in hospital settings under a doctor's direction, particularly to immunocompromised patients. Apart from the inconvenience of administration, these drugs also produce side effects [5, 15, 16]. Apart from its specific action against the influenza virus, arbidol has an immunomodulating effect, has the capability to induce interferon, and is recommended for patients with compromised immune systems [6, 7, 17].

In order to reduce the likelihood of the development of resistant strains, or increase the effectiveness of lower doses of amantadine, rimantadine, ribavirin and ribamidil, we studied the effects of combining these drugs with arbidol. Not one of the concentrations of arbidol we used weakened the effect of amantadine or rimantadine on viral reproduction. Adding amantadine 1 mcg/ml to arbidol did not have any significant influence on the inhibition degree of viral reproduction, greater than the effect of the same concentrations given alone. The combinations of arbidol and amantadine (arbidol 1 mcg/ml + amantadine 10 mcg/ml; arbidol 5 mcg/ml + amantadine in all studied concentrations; and arbidol 10 mcg/ml + amantadine in all studied concentrations) strengthened the suppressive effect of arbidol on viral reproduction, in comparison to the effect shown by the same concentrations of arbidol and amantadine alone, but the strongest inhibitory effect was observed for the combination 5 mcg/ml arbidol + amantadine in concentrations 0.3 mcg/ml or 1 mcg/ml. The data was similar for arbidol's effect on viral reproduction in experiments in combination with rimantadine (Table 4).

Table 4: The effect of arbidol in combination with various anti-influenza drugs on viral reproduction of influenza virus A/Singapore/1/57 in MDCK cell culture

arbidol, mcg/ml	% inhibition of viral reproduction									
	amantadine, mcg/ml					rimantadine, mcg/ml				
	0	0.3	1	5	10	0	0.3	1	5	10
0	n/a	22	28	50	56	38	n/a	25	31	56
1	30	30	30	52	59	30	31	33	50	59
5	38	60	68	74	81	38	63	71	74	82
10	67	77	81	82	85	67	78	80	82	86
	ribavirin, mcg/ml					ribamidil, mcg/ml				
	0	1	3	10	15	0	1	3	10	15
0	n/a	5	25	30	38	n/a	4	28	32	40
3	n/a	19	30	52	54	n/a	17	52	50	59
10	23	26	35	85	82	24	25	68	79	80
15	40	43	41	82	80	38	41	42	72	84
20	55	65	68	80	82	54	71	72	80	86

Note: n/a = not active.

The addition of ribavirin in concentrations of 1, 3 and 10 mcg/ml to all tested concentrations of arbidol increased its inhibitory effect, but the rate of increase differed among the different concentrations. The biggest increase in inhibitory effect of both preparations was observed under the following combinations: arbidol 3 mcg/ml + ribavirin 1 mcg/ml; arbidol 10 mcg/ml + ribavirin 10 mcg/ml; and arbidol 15 mcg/ml + ribavirin 10 mcg/ml. Adding ribamidil to arbidol in various concentrations also increased the inhibiting effect of arbidol on viral reproduction in every case; however, the combination of arbidol and ribamidil which resulted in the biggest increase in inhibition of viral reproduction differed somewhat from the result of the arbidol and ribavirin combination. The biggest increase in inhibiting effect of arbidol and ribamidil was observed under the following combinations: arbidol 3 mcg/ml + ribamidil 1 mcg/ml; arbidol 3 mcg/ml + ribamidil 3 mcg/ml; arbidol 10 mcg/ml + ribamidil 3 mcg/ml; and arbidol 10 mcg/ml + ribamidil 10 mcg/ml (Table 4).

Thus, in the study of the effects of arbidol in combination with other anti-influenza drugs which have a different mechanism of action from it, nowhere was observed an antagonistic reaction between the drugs. The combinations of amantadine, rimantadine, ribavirin and ribamidil with arbidol increased the effectiveness of lowered doses of these drugs, which lessens the chance of side effects from the drugs, and decreases the likelihood of the development of resistant strains from use of the adamantadine drug group.

The data concerning arbidol's lack of specificity in regards to influenza virus groups A and B; its effectiveness against strains resistant to rimantadine, and against avian influenza strains which are pathogenic in humans; and its heightened effectiveness in combination with other anti-influenza preparations in cell culture experiments – all provide a foundation for widening the possibilities for arbidol in RSV chemotherapy, and increasing arbidol's use in clinical settings.

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