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Ribavirin: Biotechnological Synthesis and Effect on the Reproduction of *Vaccinia* Virus

I. D. Konstantinova*, [1], N. A. Leont'eva, G. A. Galegov**, O. I. Ryzhova*, D. V. Chuvikovskii*, K. V. Antonov*, R. S. Esipov*, S. A. Taran***, K. N. Verevkina***, S. A. Feofanov***, and A. I. Miroshnikov***

**Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow, 117997 Russia*

***Ivanovskii Institute of Virology, Russian Academy of Medical Sciences, ul. Gamalei 16, Moscow, 123098 Russia*

****Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry (Pushchino Branch), Russian Academy of Sciences, pr. Nauki 6, Pushchino, Moscow oblast, 142292 Russia*

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Abstract—The biotechnological method of synthesis of ribavirin, vidarabin, and 6-azauridine by the use of immobilized recombinant enzymatic preparations of nucleoside phosphorylase was improved. The effect of ribavirin and its combinations with the other synthesized nucleosides on the reproduction of *Vaccinia* virus was studied on the culture of Vero cells. The combination of ribavirin and vidarabin was shown to provide the antiviral effect at lesser concentrations than with these compounds taken separately.

Key words: 6-azauridine, immobilized enzymes, purine nucleoside phosphorylase, ribavirin, uridine phosphorylase, Vaccinia virus, vidarabin

INTRODUCTION

Modified nucleosides whose structures are similar to those of intermediate products of DNA and RNA biosynthesis constitute a large group of antiviral preparations.[2] The mechanism of antiviral action of the purine and pyrimidine analogues consists in their inclusion into the structure of growing viral DNA instead of natural nucleosides in the composition of the corresponding nucleotides. In a number of cases, this results in the inhibition of DNA and RNA polymerases, stoppage of the growth of the chain of viral nucleic acid, and, consequently, the loss of its virulence.

A pronounced inhibiting effect toward the DNA-containing viruses, including the viruses of herpes simplex and pox and adenoviruses, exhibits a group of modified nucleosides: 5-bromo-2'-deoxyuridine, 1-β-*D*-arabinofuranosyladenine, and AraA; they are used in medical practice. An outstanding role in the herpes therapy play acyclic guanine-containing nucleosides, e.g., acyclovir (acycloguanosin). Ribavirin (1-β-*D*-ribofuranosyl-1,2,4-triazol-3-carboxamide) that exhibits a wide spectrum of antiviral activities toward a great number of RNA and DNA viruses is used in systemic therapy of a number of distributed viral infections of humans, such as viral hepatitis C and the respiratory-syncytial and adenoviral infections [1, 2].

The human smallpox virus does not circulate in nature for more than 20 years and is not dangerous from the epidemic and medicinal points of view. Nevertheless, the representatives of pox viruses are being interesting not only for researchers. Currently, the probability of using some single orthopox viruses highly pathogenic for humans as the weapon of aggression (bioterrorism) seems not to be purely utopian. The effective therapeutics for the orthopox virus infections are some known nucleosides: ribavirin, AraA, cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, vistide], nucleotides, isatin β-semicarbazones, and recombinant α-interferon [3–5]. The combined action of antipox-viral compounds on an adequate model of the *Vaccinia* virus is only slightly studied. We believe that the application of combinations of real antipox-viral drugs (e.g., a combined use of modified nucleosides and nucleotides and α-interferon) could be promising in the medicinal control of the human poxviral infection. Our study is devoted to studying the action of ribavirin and its combinations with AraA and

[1] Corresponding author; phone: +7 (095) 330-7247; e-mail: kid@kou.ibch.ru

[2] Abbreviations: AraA, vidarabin (9-β-*D*-arabinofuranosyladenine); AzUr, 6-azauridine; CE_{50}, 50% cytotoxic infectious dose for the noninfected Vero cells; CEF, chicken embrio fibroblast; CPE, viral cytopathogenic effect; IE_{50} and IE_{90}, doses of preparations inhibiting viral cytopathogenic effect by 50 and 90%; PFU, plaque-forming unit; Puo-phosphorylase, a purine nucleoside phosphorylase obtained using the genetic engineering overproducing *Escherichia coli* strain BL21(DE3)/pERPUPHO1; TCA, 1,2,4-triazol-3-carboxamide; $TCID_{50}$, 50% tissue cytotoxic infectious dose; and Urd-phosphorylase, uridine phosphorylase from the genetic engineering overproducing *E. coli* strain BL21(DE3)/pERUPHO1.

AzUr on the reproduction of *Vaccinia* virus in the Vero cell culture.

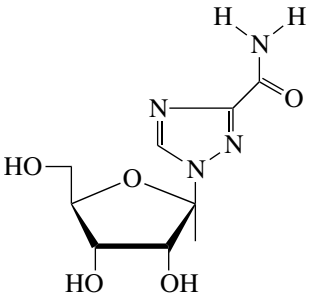
A steady growth and occurrence of human viral diseases and the practically proven efficiency of the medicinal use of medicines on the basis of modified nucleosides provide for a high interest to the development of modern biotechnological methods of manufacturing of antiviral preparations of a new generation for their wide use in practical medicine.

In this connection, we describe in this work an improved highly efficient method of the preparation of ribavirin and some other modified analogues of nucleosides based on the reaction of microbiological transglycosylation by means of immobilized nucleoside phosphorylases obtained by genetic engineering.

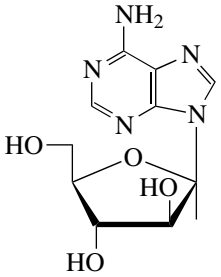
RESULTS AND DISCUSSION

A Biotechnological Method of Obtaining Modified Nucleosides

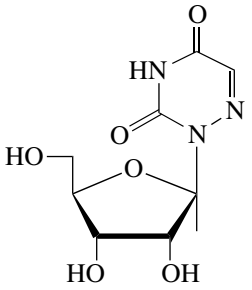
The modified nucleosides ribavirin, AraA, and AzUr are used in the modern medicinal practice for the combined therapy of viral infections and the treatment of particularly dangerous diseases of humans, such as bronchiolitis in children of the first life year, various forms of herpetic illnesses, and chronic hepatitis C.



Ribavirin



AraA



AzUr

The chemical synthesis of modified nucleosides is a complex and multistage process [6–9], the key stage of which is the glycosylation of a modified nucleic base with a protected derivative of ribose (arabinose). The performing of this reaction with selective chemical methods leads to the formation of a racemic mixture of α and β anomers with 90–95% predominance of the natural β anomer. The separation of minor impurities of the unnatural α anomer is a rather laborious process, which is also complicated by the necessity of removal of protective groups. Therefore, the number of stages in the chemical methods of synthesis of modified nucleosides is varied on the average from five to nine, and the total yields of the target products are consequently reduced.

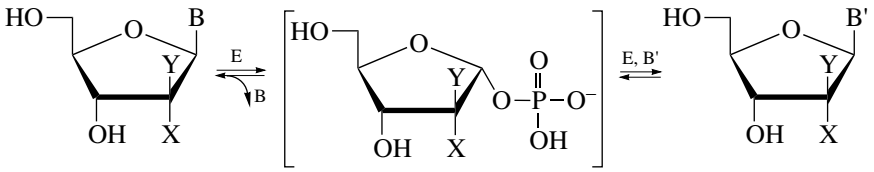
An alternative method of the synthesis of natural and modified nucleosides is based on the reaction of microbiological transglycosylation, which is achieved by the use of nucleoside phosphorylases (Scheme 1) [10–13]. A transfer of a carbohydrate residue from a natural purine or pyrimidine nucleoside to a natural or a modified heterocyclic base occurs during the reaction.

All the known works devoted to the preparation of modified nucleosides by chemoenzymatic methods used bacterial strains of microorganisms, obtained by a microbiological selection, as a source of nucleoside phosphorylases. It is the intact cells of these microorganisms of the enzymes themselves isolated from the bacteria that served as catalysts of the biochemical transformations [10–15].

In this work, we improved and optimized the technological scheme of preparation of the preparations of ribavirin, AraA, and AzUr using the previously developed genetic engineering nucleoside phosphorylases: Puo-phosphorylase from the overproducing *Escherichia coli* strain BL21(DE3)/pERPUPHO1 (EC 2.4.2.1) [16, 17] and uridine phosphorylase (Urd phosphorylase) from the overproducing *E. coli* strain BL21(DE3)/pERUPHO1 (EC 2.4.2.3) [18]. They were immobilized on an aminopropylated macroporous glass AP-CPG-170 (Sigma, United States). The use of these immobilized enzymatic preparations significantly simplifies the technology of preparation of the modified nucleosides and allows a multiply repeated use of the catalytic activity of the enzymatic preparations (up to 15 manufacturing cycles).

The enzymes were immobilized on the aminopropylated macroporous glass AP-CPG-170 by glutaric dialdehyde by the method [19]. A covalent bond between the aldehyde groups and the amino groups of the sorbent and proteins is formed in the presence of sodium borohydride [20].

The synthesis of ribavirin was further carried out by Scheme 2. The presence of inorganic phosphate is necessary in the reaction of enzymatic transglycosylation for the efficient formation of the intermediate α-*D*-ribosyl-1-phosphate [11]. The optimal conditions for the reaction are: 60°C and pH 7.0. The characteristics of the transglycosylation reaction in the synthesis of modified



where X = OH, Y = H, B = Gua, B' = 1,2,4-triazol-3-carboxamide;
X = H, Y = OH, B = Ura, B' = Ade;
X = OH, Y = H, B = Ade, B' = 6-azauracil;
E – Puo/Urd phosphorylases.

Scheme 1.

nucleosides are more comprehensively described in Table 1.

We demonstrated a possibility of obtaining the modified nucleosides AraA and AzUr using a similar scheme and a binary system of immobilized Puo- and Urd-phosphorylases.

Thus, an efficient biotechnological method of preparation of a number of modified nucleosides using nucleoside phosphorylases obtained by genetic engineering and immobilized on an aminopropylated macroporous glass. The simplicity and versatility of this approach allow scaling up the process and prepare development batches of modified nucleosides for biological testing.

The results allow one to predict a basic possibility of the development of efficient contemporary technological sets for the synthesis of a number of modified nucleosides with the use of immobilized nucleoside phosphorylases obtained by genetic engineering.

The Study of Antiviral Activity of Modified Nucleosides

The results of studying the effects of the synthesized ribavirin, AraA, and their combinations on the reproduction of *Vaccinia* virus (the infection titer of the virus, logBFU/ml, was determined; see figure) demonstrated that the combination of the two modified nucleosides is effective and a synergic antiviral effect was observed even after a four times decrease in the ribavirin concentration and two times decrease in AraA concentration.

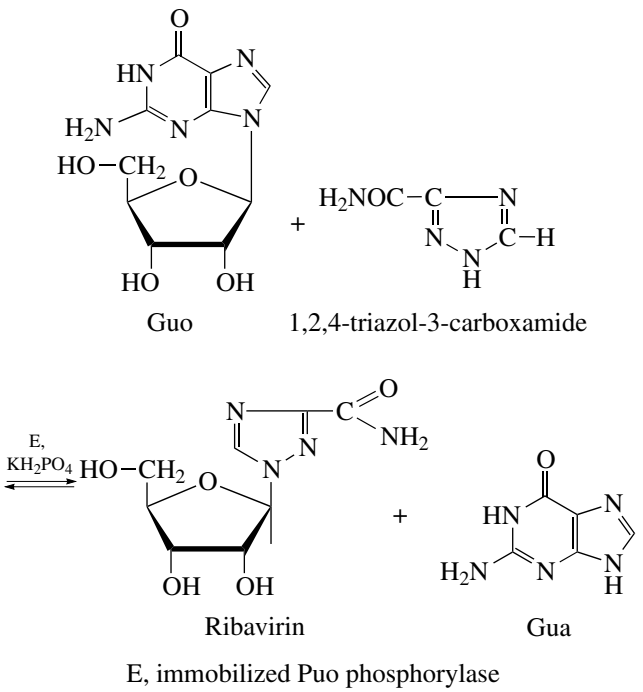
The development of the viral infection was determined from the value of virus-induced CPE (Table 2). The antiviral activity of the studied compounds (individually or in combination) was evaluated according to their ability to inhibit the development of the virus-induced CPE by 50% (IE_{50}) and almost completely (IE_{90}). Table 2 shows that the three modified nucleosides protect cells and prevent the development of CPE, although their combinations inhibit CPE at lower concentrations. This is also true in respect of the *Vaccinia* virus resistant to 5-iodo-2'-deoxyuridine. The combinations of ribavirin and AraA turned out to be the most effective, whereas the efficiencies of AzUr and AraA combinations were less pronounced. On the basis of the mechanism of action of AraA and its 5'-phosphate, one could conclude that the reproduction of *Vaccinia* virus could be destroyed directly at the stage of synthesis of viral DNA [21]. At the same time, literature data suggest that ribavirin (in the form of its 5'-triphosphate) decreases the translation efficiency of mRNA of the

Table 1. The characteristics of transglycosylation reaction in the synthesis of modified nucleosides

Substrates for enzymatic reaction (60°C, pH 7.0)	Immobilized enzyme	Reaction time, days	Content of the target product*, %	Yield**, %
Guo (60 mM) TCA (40 mM) KH_2PO_4 (50 mM)	Puo-phosphorylase	1.5	Ribavirin, 92	68
6-Azauracil (3 mM) Adenosin (10 mM) KH_2PO_4 (0.4 mM)	Puo/Urd-phosphorylases	0.2	6-Azauridine, 19.3	45
1-β-*D*-Arabinofuranosyluracil (5 mM) Adenine (6 mM) KH_2PO_4 (5 mM)	Puo/Urd-phosphorylases	1	Vidarabin, 45.7	56

* HPLC-monitoring of incubation mixture.

** After purification (see the Experimental section).



Scheme 2.

Vaccinia virus, because it precludes the formation of cap-structure at the 5'-terminus of its RNA [22–24].

We also obtained a positive antiviral effect for the ribavirin–AraA combination on the primary culture of CEF. At the multiplicity of infection of 0.1 BFU per cell and the introduction of the compound directly after the infection of cells, we obtained the following results (see Table 3).

It was shown that ribavirin biotechnologically obtained in IBCh RAS efficiently inhibits the reproduction of *Vaccinia* virus in the Vero and CEF cell cultures at both a high and a low multiplicity of infection. The combination of ribavirin and AraA provides an intensification of the antiviral effect at lower concentrations than those of individual compounds. Ribavirin and AraA effectively inhibit the reproduction of the *Vaccinia* virus variant resistant to 5-iodo-2'-deoxyuridine. This effect is also retained at the use of combinations of these modified nucleosides at lower concentrations. It is real to use a combination of the two compounds with different mechanisms of action on the reproduction of *Vaccinia* virus also for the possible treatment of orthopox-viral infection of humans.

A high sensitivity of the reproduction of pox viruses to α-interferon makes it topical to study its antiviral activity in combination with ribavirin and AraA. This might be an optimal scheme of treatment of the pox-viral infection in humans. At the same time, a combination of α-interferon and ribavirin has been turned out to be efficient at the treatment of the widely distributed viral hepatitis C; this combination could also be promising for the therapy of the recent outbreak of the RNA-viral infection of atypical pneumonia induced by a crown virus.

EXPERIMENTAL

We used in this work domestic solvents and reagents as well as some reagents from Sigma (United States) and Chemapol (Czech Republic) and guanosine form the Novopolotsk BVK plant (Belarus) without additional purification.

Substances were identified and their purity was checked by TLC on the plates Kieselgel 60 F_{254} and HPTLC Kieselgel 60 plates (Merck, Germany) in the system 9 : 1 acetonitrile–water; spots were detected by spraying with 70% H_2SO_4 followed by heating.

The homogeneity of substances, reaction course, and yields of products were monitored by HPLC on a Waters 740 chromatograph using an Altima (0.4 × 25 cm) column filled with Nucleosil C_{18} 5 μm sorbent.

Table 2. The development of the virus-specific CPE in the Vero cell cultures infected with the *Vaccinia* virus in the presence of ribavirin, AraA, AzUr, and their combinations*

Series	Compound	IE_{50}, μg/ml		IE_{90}, μg/ml		CE_{50}, μg/ml
		VV	VV-R	VV	VV-R	
I	Ribavirin	3.0	3.0	3.0	6.0	95
II	AraA	2.0	2.5	5.0	5.0	37
III	AzUr	20	20	–**	–**	90
IV	Ribavirin/AraA	0.22/0.22	0.22/0.5	0.5/0.5	0.5/0.5	≥>50/10
V	Ribavirin/AzUr	3.0/3.5	3.0/3.5	7.5/7.0	7.5/7.0	>25/25
VI	AraA/AzUr	3.75/1.0	3.75/1.5	7.5/2.0	7.5/3.0	>25/10

*Result 72 h after the cell infection. The multiplicity of infecting is 0.001 PFU/cell. VV, *Vaccinia* virus; VV-R, *Vaccinia* virus resistant to 5-iodo-2'-deoxyuridine.

**Effect was not achieved.

Elution was carried out at a rate of 1 ml/min in the following solvent systems. *System 1*: water as solution A and 1 : 1 acetonitrile–water as solution B, gradient of B in A from 0 to 100% for 40 min, was used in the analysis of ribavirin synthesis at the detection at 214 nm. *System 2*: water as solution A and 7 : 3 acetonitrile–water as solution B, 7 min at 0% B and gradient of B in A from 0 to 100% for 40 min, was used in the analysis of AzUr synthesis at the detection at 254 nm. *System 3*: water as solution A and 7 : 3 acetonitrile–water as solution B, gradient of B in A from 0 to 100% for 40 min, was used in the analysis of AraA synthesis at the detection at 254 nm.

Ion exchange chromatography was carried out using a column (3 × 9 cm) filled with Dowex 1x8 (OH^-), 20–50 mesh) and a column (2.2 × 12 cm) with Dowex 50x8 (H^+, 400 mesh, Serva).

Column chromatography was performed on C_{18} Octadecyl=Si 100polyol (0.03 mm) sorbent (Serva, United States).

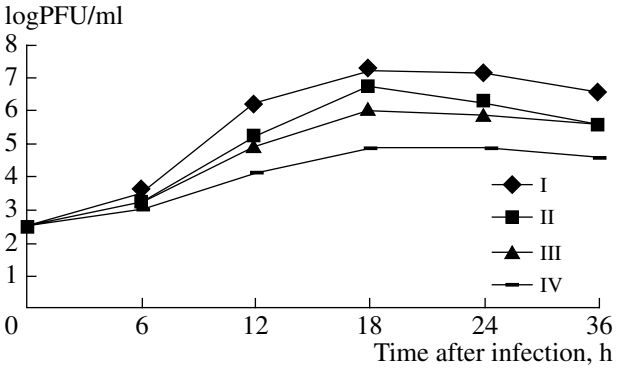
UV spectra were measured on a UV-160 Shimadzu spectrophotometer (Japan), and IR spectra, on a Hitachi 270-30 spectrophotometer. ^1H NMR spectra were registered on a Bruker DRX-500 instrument in D_2O. Mass spectra (ESI) were obtained on a Finnigan 900 S spectrometer.

Immobilized Enzymic Preparations

An aminopropylated macroporous glass AP-CPG-170 (10 g, 125–225 μmol of primary amino groups per g, 200–400 mesh) was successively washed with distilled water (40 ml), ethanol (80 ml), distilled water (40 ml), and 0.1 M potassium phosphate (pH 7.3, 80 ml). The prepared sorbent was wrung out from the buffer on a glass filter, mixed with 5 vol % glutaric dialdehyde solution in 0.1 M potassium phosphate (pH 7.3, 250 ml), stirred for 10 h, and wrung out from the reaction mixture on a glass filter and washed with 0.1 M potassium phosphate (pH 7.3, 80 ml). The sorbent was then wrung out on a glass filter and weighted. The wet sorbent was prepared immediately before the application of enzymic preparations and used for a day.

A lyophilized powder of Puo-phosphorylase obtained from an overproducing strain BL21(DE3)/pERPUPHO1 of *E. coli* [16, 17] has a specific activity of 20 U/mg of protein; 1 U of the enzyme converts 1 μmol of inosine into hypoxanthine in 50 mM potassium phosphate, pH 7.3, for 1 min at 37°C. The Puo-phosphorylase preparation was dissolved in 50 mM KH_2PO_4 buffer, pH 7.3, to get 30 ml of a solution with the protein concentration of 5 mg/ml (determined by the Lowry method [25]).

A solution of Puo phosphorylase was added to a wet sorbent, and the mixture was kept on a shaker for 18–21 h at 4°C, avoiding the formation of foam and monitoring the protein concentration in supernatant by taking 100-μl aliquots.



Effect of ribavirin, AraA, and their combinations on the reproduction of *Vaccinia* virus in the Vero cell culture. Infection multiplicity 5 PFU/cell. The preparations were added at the following concentrations (μg/ml): I, 0; II, ribavirin (20); III, AraA (5); IV, ribavirin (5) + AraA (2.5).

When the protein content in supernatant became ≤0.5 mg/ml, the sorption was stopped, the sorbent was three times washed successively with 50 mM potassium phosphate (pH 7.3, 60 ml) and 2 M KCl (20 ml) and treated with a solution (20 ml) of sodium borohydride (90 mg) in distilled water for 1 h at 4°C. Then the sorbent was three times successively washed with 50 mM potassium phosphate (pH 7.3, 60 ml) and 2 M KCl (20 ml), the quantity of nonsorbed protein was determined in the washings, and the quantity of protein immobilized on 1 g of sorbent was calculated as a difference.

The yield of wet sorbent was 30 g, and it contained 90 mg of Puo phosphorylase (3 mg of protein per 1 g of wet sorbent from the results of three experiments).

The enzymatic activity of the recombinant immobilized Puo-phosphorylase was determined according to its ability to cleave inosine to hypoxanthine. The quantity of enzyme capable of the conversion of 1 μmol inosine into hypoxanthine for 1 min at 37°C was taken

Table 3. The effect of ribavirin, AraA, and their combinations on the reproduction of *Vaccinia* virus in the CEF cell culture

Compound	Concentration, μg/ml	Infection titer of virus, logPFU/ml, after 48 h*
(control)	–	7.4
Ribavirin	15	6.6
	25	6.0
AraÄ	3	6.3
	6	5.7
Ribavirin + AraÄ	10 + 2	5.2
	12.5 + 3	4.2

* Multiplicity of infection is 0.1 PFU/cell. Compounds were added immediately after the infection of cells.

as 1 U. The specific activity of the immobilized Puo-phosphorylase under these conditions was no less than 15 U/mg of the immobilized protein.

The preparation of Puo-phosphorylase was stored at –4°C no longer than four weeks.

The immobilized Urd-phosphorylase was similarly prepared from the enzyme isolated from the *E. coli* overproducing strain BL21(DE3)/pERUPHO1 [18]. The specific activity of the immobilized Urd-phosphorylase was no less than 25 U/mg of the immobilized protein.

1-β-*D*-Ribofuranosyl-1,2,4-triazol-3-carboxamide. A mixture of guanosine (17.0 g, 0.06 mol) and 1,2,4-triazol-3-carboxamide (4.48 g, 0.04 mol), and immobilized Puo-phosphorylase (30 g) was incubated in 50 mM KH_2PO_4 buffer (pH 7.0, 1 l) for 36 h at 60°C. The process was monitored by HPLC; it was approved to be complete when the ribavirin content in the reaction mixture reached 86–92%. The immobilized enzymic preparation was separated from the reaction mixture by decantation, washed with 50 mM KH_2PO_4 (pH 7.0, 60 ml), and repeatedly used for the preparation of ribavirin no less than 15 times. The reaction solution was cooled to +4°C, and the precipitated guanine was separated by filtration and washed with water (30 ml). The filtrate was evaporated in a vacuum to a small volume and passed through a layer of Dowex 50 (H^+) to remove the traces of heavy metals and inorganic cations; the crude ribavirin was washed out with water. The eluate was evaporated in a vacuum to a small volume, and passed through a layer of Dowex 1 (OH^-) to purify from the admixtures of starting compounds (guanosine and 1,2,4-triazol-3-carboxamide), inorganic phosphate, and traces of guanine. Ribavirin was eluted with water, and the resulting aqueous solution was evaporated in a vacuum to dryness (55°C). The residue was dried in a desiccator to a constant weight, and the product was crystallized from ethanol with water added (up to 10 vol %) to give a white crystalline powder, which was dried at 100°C for 5 h. The yield of the product was 6.60 g (68%); the content of the main substance 99.9% (HPLC, system 1); $[\alpha]_D^{20}$ –36.2 (*c* 1, H_2O); mp 168–170°C (lit. data: mp 166–168°C [6]); R_f 0.48; λ_{max}, nm (ε, M^{-1} cm^{-1}): 205.6 (11 300–11 400); MS, *m/z*: 245.5 $[M + H]^+$, 267.1 $[M + Na]^+$, 282.9 $[M + K]^+$, 487.9 $[2M]^+$, 510.0 $[2M + Na]^+$, 526.0 $[2M + K]^+$; ^1H NMR (D_2O, δ, ppm, *J*, Hz): 8.80 (1 I, br. s, H5), 6.20 (1 I, d, $J_{1',2'}$ 3.44, H1'), 4.71 (1 I, dd, $J_{2',1'}$ 3.44, $J_{2',3'}$ 5.27, H2'), 4.55 (1 I, dd, $J_{3',2'}$ 5.27, $J_{3',4'}$ 5.27, H3'), 4.28 (1 I, dt, $J_{4',3'}$ 5.27, $J_{4',5a'}$ 3.21, $J_{4',5b'}$ 5.27, H4'), 3.93 (1 I, dd, H5*a*', $J_{5a',5b'}$ 12.6, $J_{5a',4'}$ 3.21, H5a'), and 3.82 (1 I, dd, $J_{5b',5a'}$ 12.6, $J_{5b',4'}$ 5.27, H5b').

AzUr. A mixture of adenosine (0.53 g, 1.9 mmol) and 6-azauracil (0.068 g, 0.06 mmol), and immobilized Puo- (2 g) and Urd-phophorylases (3 g) in 0.4 mM potassium phosphate buffer (pH 7.0, 0.2 l) was stirred for 4.8 h at 60°C. The process was monitored by HPLC; it was considered to be complete at the AzUr content in the reaction mixture of 18–19%. The reaction mixture was separated from the immobilized enzymic preparation by decantation, and the sorbent with the immobilized enzymes was washed with 50 mM potassium phosphate buffer (pH 7.0, 30 ml) and repeatedly used in the process of AzUr preparation. The solution was evaporated in a vacuum to a small volume and passed through a layer of sorbent C_{18} Octadecyl=Si 100polyol (0.03 mm). AzUr was eluted with water. The fractions containing more than 95% of the target product were collected and evaporated in a vacuum to dryness, dried in desiccator to a constant weight, and crystallized from ethanol. The white crystalline powder was dried at 50°C for 5 h, to get AzUr,; yield 66 mg (45% from 6-azauracil); the content of the main substance 99% (HPLC in system 2 and a comparison with an independent standard); mp 159–161°C; λ_{max}, nm (ε, M^{-1} cm^{-1}): 262.3 (6 100).

AraA. A mixture of adenine (0.162 g (1.2 mmol), 1-β-*D*-arabinofuranosyluracil [26] (0.24 g, 1.0 mmol), and immobolilized Puo- (2 g) and Urd-phophorylases (3 g) in 5 mM potassium phosphate buffer (pH 7.0, 0.2 l) was stirred for 24 h at 60°C. The process was monitored by HPLC; it was regarded as complete at the AraA content in the reaction mixture of 45–46%. The immobilized enzymic preparation was separated from the reaction mixture by decantation, washed with 50 mM potassium phosphate buffer (pH 7.0, 30 ml) and repeatedly used in the process of AraA preparation. The reaction solution was evaporated in a vacuum to a small volume and passed through a layer of sorbent C_{18} Octadecyl=Si 100polyol (0.03 mm). AraA was eluted with water. The fractions containing more than 95% of the target product were collected and evaporated in a vacuum to dryness, dried in desiccator to a constant weight, and crystallized from methanol. The white crystalline powder was dried at 50°C for 5 h, to get AraA; yield 0.15 g (56% from 1-β-*D*-arabinofuranosyluracil); the content of the main substance 98% (HPLC in system 3 and a comparison with an independent standard); mp 257–258°C; λ_{max}, nm (ε, M^{-1} cm^{-1}): 259 (13 100).

Antiviral Activity of Ribavirin and Other Modified Nucleosides

The antiviral activity was studied using the biotechnological ribavirin preparation from IBCh RAS obtained by the above-described procedure with the use of immobilized enzymes. In some experiments, ribavirin from ICN Pharmaceutical (United States) was used as a standard for comparison. AzUr was obtained from the Institute of Organic Chemistry (Prague, Czech Republic); AraA and 5-iodo-2'-deoxyuridine, from Sigma (United States).

Cells. A transferable culture of the Vero cells (the cells from kidney of African green marmosets) was used. They were grown on a Eagle's support medium MEM containing 10% fetal calf serum (PANEKO, Russia). The cells were cultured in 96-well plastic plates (Linbro, Flow Lab., UK) in an atmosphere enriched with CO_2.

Virus. The strain 113 obtained from the Virus Museum of the Ivanovskii Institute of Virology of the Russian Academy of Medical Sciences, Moscow.

The multiplicity of infecting cells is indicated for each experiment in tables and figure.

The inhibition of the *Vaccinia* virus reproduction in the Vero cell culture was evaluated according to the value of the infection titer of the virus (logPFU/ml) (see figure). Ribavirin was added to cells before infecting; its concentration was 10 μg/ml in series II and IV. After 45 min, the support medium was removed in series I–IV, the cells were infected (the multiplicity of infection was 5 PFU/cell), and immediately after this, the preparations were added at the following concentrations (μg/ml): I, 0 (control); II, ribavirin, 20; III, AraA, 5; and IV, ribavirin, 5 + AraA, 2.5.

The antiviral action was determined by the method [27] according to the ability of the studied compounds to inhibit the development of virus-induced CPE maximally 72 h after infecting the cells (Table 2). The compounds were added (separately or in combination) at the concentrations (μg/ml): ribavirin, 5; AraA, 2; and AzUr, 10. Support medium was removed after 60 min, cells were infected with the virus (the multiplicity of infection was 0.001 PFU/cell), and the substance under study was added immediately after this to the support medium. The effective antiviral concentrations of the compounds and their combinations were determined in all six series of experiments in comparison with the complete CPE in the control infected cultures; IE_{50} and IE_{90} were calculated.

The *Vaccinia* virus variant resistant to 5-iodo-2'-deoxyuridine (VV-R) was obtained by fivefold serial passages of the virus in the presence of increasing concentrations of this inhibitor [28].

The cytotoxicity of cells was determined by a standard method using the ability of dead cells to be stained by Trypan Blue dye [4]. The concentration that provided 50% survival of noninfected Vero cells after 72-h contact with the tested compounds was taken as the CD_{50} value (Table 2).

The inhibition of the *Vaccinia* virus reproduction in the culture of CEF cells was determine according to the value of the infection virus titer (logPFU/ml) 48 h after the infection (Table 3). The multiplicity of infection was 0.1 PFU/cell). Ribavirin, AraA, and their combinations were added immediately after the cell infection at the concentrations indicated in Table 3.

The efficiencies of the IBCh ribavirin preparation and the preparation from ICN Pharmaceutical toward the influenza A infection in the MDCK (strain H3N2/Aichi/68) cell culture were determined. The support medium for the virus-infected cell culture included trypsin. Both ribavirin preparations reduced the influenza virus reproduction within the range of noncytotoxic concentrations 6–25 μg/ml by 2.15–3.1 $logTCID_{50}$/ml.

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