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Antiviral activity of histochrome preparation

Based on the results of the MTT analysis, Histochrome® possesses antiviral activity against both tick-borne encephalitis virus (TBEV) and herpes simplex virus type 1 (HSV-1). Its IC_{50} against TBEV and HSV-1 at an infecting dose of 10^2 $TCID_{50}$/ml were 21.8±2.6 μg/ml and 18.8±2.1 μg/ml, respectively ($p \leq 0,05$). When evaluating its effect at different stages of the development of these viruses, it was shown that the drug is more effective at the early stages of the virus life cycle.

Key words: antiviral activity, tick-borne encephalitis virus, herpes simplex virus type 1, Echinocrome A.

INTRODUCTION

The increase in the share of viral infections among total infectious morbidity of the population reaches 90%, and the lack of vaccines and antiviral drugs against many viral infections is one of the most serious problems of modern healthcare. Viral diseases are a threat to public health all over the world. RNA- and DNA- containing viruses cause a number of serious animal diseases and are the most dangerous for humans. The danger of epidemics and pandemics caused by RNA- and DNA-containing viruses makes the methods of their inactivation and development of new antiviral medicines one of the most urgent tasks today. At the same time, success in the search for effective antiviral therapeutic drugs is not as significant as in the development of antimicrobial agents. [12, 11].

A number of chemical compounds that differ in the mechanisms of action, toxicity and efficiency of virus inactivation are used to treat virus infections [4]. The most difficult task is to create drugs that selectively suppress virus reproduction and do not affect the processes of cell vitality and organism as a whole. Most antiviral drugs that inhibit virus-specific processes, closely related to metabolism, energy metabolism and enzymatic reactions in the cell, almost always have a toxic effect on the cell itself. As a rule, the drugs available in therapeutic practice have rather low efficacy, even if they are used at an early stage of the disease. They have a narrow spectrum of action (one virus/one drug) and pathogenic viruses often acquire resistance to such drugs.

For example, an antiviral agent acyclovir (2-amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6H-purine-6-one) which is an analogue of the purine nucleoside deoxyguanosine, a normal DNA component, is known. Acyclovir is an effective drug against herpes simplex virus type 1 (HSV-1), herpes zoster (lichen) and chicken pox. In cell culture acyclovir has the most pronounced antiviral activity against HSV-1, HSV-2, *Varicella zoster*, Epstein-Barr and cytomegalovirus [10]. Prolonged treatment with this drug leads to impaired renal function and causes acute renal failure.

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An antiviral agent of plant origin panavir is a high molecular weight polysaccharide [18]. It increases the nonspecific resistance of the organism to various infections, strengthens the immune system, promote induction of interferon synthesis. This drug is used in the treatment of herpes virus infections of various locations, as well as in the treatment of tick-borne encephalitis virus (TBEV). The disadvantage of the drug panavir is the lack of virucidal action [7, 15].

The preparations which have antiviral activity against TBEV are 7,3'-luteolin disulfate obtained from the water-ethanol extract of marine herbs of the family Zosteraceae [18], iodantipyrine, based on 1-phenyl-2,3-dimethyl-iodopyrazolone-5 [19], as well as exopolysaccharide from marine bacteria *Pseudoalteromonas nigrifaciens* strain KMM 156 [21]. However, the above drugs, having different mechanisms of action, do not have antiviral activity against HSV-1. Chemotherapy drugs based on oxolin (2,2,3,3-tetrahydroxy-2,3-dihydro-1,4-naphthoquinone) have a virucidal effect on influenza viruses, HSV-1, adenoviruses of infectious warts. They are also used to prevent and treat respiratory viral infections [1, 20, 8]. However, oxolin is not effective enough against HSV-1 and TBEV [1, 20]. Consequently, the expansion of the arsenal of effective and low-toxic agents for the prevention and treatment of TBEV and HSV-1 is currently an urgent task. Thus, the search for non-toxic effective antiviral drugs with a wide spectrum of action is of great importance.

In order to search for new broad-spectrum antiviral agents, we studied Histochrome® as a new agent both against TBEV (RNA virus) and HSV-1 (DNA virus), and also evaluated its influence at different stages of viruses' development.

The drug Histochrome® is a dosage form of an individual substance - a quinonoid pigment of sea urchins echinochrome A (2,3,5,6,8-pentahydroxy-7-ethyl-1,4-naphthoquinone) (number of state registration P N002362/01-2003).

Histochrome is used for the treatment of eye and heart diseases. Histochrome in the form of injection solution (0.02 mg/ml) is used to treat dystrophic retina and corneal diseases, retinal diabetic retinopathy, vitreous hemorrhage, anterior chamber, dyscirculatory disorders in the central artery and retinal vein (state registration number P N002363/02-2003) [6].

A solution for intravenous administration of 10 mg/ml histochrome is used in cardiology after acute myocardial infarction in combination with thrombolytic drugs to reduce the size of myocardial infarction and to prevent reperfusion injury of the myocardium (registration number P N002363/01-2003) [9, 5]. These inventions are also patented in the USA (US 6384084 B2, 07.05.2002, US 6410601 B2, June 25, 2002) and in the countries of the European Union (EP 1121929 A1, 08.08.2001, EP 1121930, A1, 08.08.2001). New applications of histochrome for the treatment of hemorrhagic stroke, of cerebral ischemia and acute disorders of cerebral circulation [17], as a diuretic agent [13] were patented.

The use of histochromeas an antiviral agent have not been described in patent, scientific and technical literature so far. The antiviral activity of histochrome against the most common flaviviruses in the Russian Federation – TBEV, as well as against HSV-1 was revealed and was determined experimentally by authors for the first time.

METHODS

Viruses and cell cultures.

RNA-containing tick-borne encephalitis virus (TBEV) (Dal'negorsk strain of the Far Eastern subtype) was isolated in the laboratory of flaviviruses infections of the Somov Institute of Epidemiology and Microbiology in 1973 from the brain of a dead patient who had the focal form of TBE. (Gene Bank Whole Genome Sequence Number: FJ402886) [3, 14]. A 10% virus-containing suspension of the brain of suckling mice infected with this strain (10 passage) was used. The TBEV titer was $10^{8.8}$ $TCID_{50}$/ml. The DNA-containing herpes virus (HSV-1, strain VR3) was obtained from the National Collection of US Viruses (Rockville, Maryland, USA).

The strain of HSV-1 passed 5-7 consecutive passages on Vero cell culture. The titer of HSV-1 was $10^{8.25}$ $TCID_{50}$/ml.

The study of the antiviral activity of drugs against TBEV was carried out on the PK (pig embryo kidney) cells grown using medium 199 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml gentamicin at 37°C in a CO_2 incubator, in the maintenance medium the concentration of FBS was reduced to 1%. Studies of the anti-herpetic activity of drugs were carried out on the Vero (African green monkey kidney) cells. The cells were grown using a complete DMEM culture medium supplemented with 5-10% FBS, 0.008% solution of gentamicin sulfate and glutamine at 37°C in a CO_2 incubator. In all experiments, the cell concentration was 10^4 cells/ml.

Studied preparations:

Histochrome® – a solution of 10 mg/ml in an ampoule (manufactured by PIBOC FEB RAS).

Oxolin® (Biosynthesis, Russia);

Placebo – a composition of antioxidants containing ascorbic acid (99.8%, pharmaceutical, AppliChem, Germany) and α-tocopherol (≥96%, Ph. Eur. Carl Roth, Germany) at a 5:1 weight ratio.

The tested preparations, except histochrome, were dissolved in dimethylsulfoxide (DMSO, Sigma, USA) and stored at -20 °C. The stock solutions (10 mg/ml) of preparations were diluted with a suitable cell culture medium so that the final concentration of DMSO was 0.5%.

Determination of cytotoxic activity.

The cytotoxicity of the preparations was evaluated by the viability of the PKV and Vero cells using the MTT assay [2, 16]. A monolayer of cells (2×10^4 cells/well) grown in 96-well plates was treated with various concentrations (0 to 400 μg/ml) of tested preparations and untreated cells as control. The cells were cultured at 37 °C in a CO_2 incubator for 6 days. After incubation, 20 μl/well of a solution of MTT (methylthiazolyltetrazolium bromide, Sigma, USA) at a concentration of 5 mg/ml was added to the monolayer of cells, left for 2 hours at 37 °C., then isopropyl alcohol acidified with 0.4 M HCl (150 μl/well) was added. The optical density (OD) was measured at 540 nm on a 96-well reader (Labsystems Multiskan RC, Finland). The viability of the cells was calculated as (ODt)/(ODc) × 100%, where ODt is the optical density of cells treated with the tested compounds, OD_C is the optical density of the untreated cells. A value of 50% of the cytotoxic concentration (CC_{50}) was determined by regression analysis as the concentration of the drug, which reduced the number of viable cells by 50% compared to cell control.

Determination of antiviral activity.

Antiviral activity was determined on the basis of MTT assay by inhibiting the cytopathic effect of the virus using inverted microscope (Biolam P-1, LOMO, Russia) [2, 16]. The preparations were tested in the concentration range from 0 to 400 μg/ml and at several infectious doses of the virus (from 10 to 10^3 $TCID_{50}$/ml). Each infectious dose of the virus was combined with various concentrations of compounds at a ratio of 1:1, incubated for 1 h at 37 °C. Then, it was applied to a monolayer of cells (2×10^4 cells/well) grown in 96-well plates and cultured for 6 days at 37 °C in a CO_2 incubator.

The antiviral activity of the preparations (for each infectious dose of the virus) was assessed by the inhibition rate (IR) of the virus by the drug, 50% inhibitory concentration (IC_{50}), and the selective index (SI).

IR was calculated by the formula IR = (ODtv−ODcv)/(ODcd−ODcv)×100%. ODtv indicates the absorbance of the tested preparations with virus infected cells. ODcv and ODcd indicate the absorbance of the virus control and the absorbance of the cell control, respectively.

IC_{50} was determined by regression analysis of the dependence of the virus inhibition rate (IR) in % on the drug concentration, as the concentration of the drug that inhibited the cytopathic effect of the virus by 50% compared to the control.

The selectivity index (SI), the therapeutic index of the preparation, was calculated as the ratio of CC_{50} to IC_{50}.

Virucidal activity of tested preparations.

The viruses were treated with a solution of the tested preparations at a ratio 1:1, incubated for 1 h at 37 °C, then applied to a monolayer of cells and cultured for 6 days at 37 °C in a CO_2 incubator.

Preventive activity of tested preparations.

The monolayer of the cells was treated with the tested preparations for 1 h at 37 °C., then infected with the virus and cultured for 6 days at 37 °C in a CO_2 incubator.

Virus-inhibiting activity of the tested preparations.

The monolayer of the cells was infected with the virus and kept for 1 h (for the TBEV virus) or 10-15 minutes (for the HSV-1 virus) at 37 °C. The cells were then treated with test preparations and cultured for 6 days at 37 °C in a CO_2 incubator.

RESULTS

Antiviral activity of the tested preparations against tick-borne encephalitis virus.

Based on the results of MTT assay, 50% of the cytotoxic concentration (CC_{50}) was calculated for each preparation which treated PK cells. The main indicators of antiviral activity against various infectious doses of tick-borne encephalitis virus are presented in Table 1.

As we can see from Table 1, oxolin and placebo were less cytotoxic to PK cellsthan histochrome ($p \leq 0.05$). However, the histochrome drug exhibits a higher antiviral activity against the TBEV virus than the reference drugs. Thus, suppression of viral replication with all infectious doses occurs at significantly lower inhibitory concentrations (IC_{50}) histochrome than oxolin and placebo, and therefore the selective index (SI) of histochrome characterizing the efficacy of the drug is significantly higher than those for oxolin and placebo ($p \leq 0.05$).

Table 1

Antiviral activity of the tested preparations against tick-borne encephalitis virus

Preparation	Cytotoxicity CC_{50}, μg/ml	Infectious dose ($TCID_{50}$/ml)	Inhibiting concentration IC_{50}, μg/ml	Selectivity index (SI)
Histochrome	54.4±1.8	10^1	10.7±1.2	5.2±0.5
		10^2	21.8±2.6	2.5±0.2
		10^3	39.8±5.2	1.3±0.1
Oxolin	104.6±6.1*	10^1	74.5±8.2*	1.4±0.2*
		10^2	95.1±10.0*	1.1±0.1*
		10^3	174.3±19.4*	0.6±0.1*
Placebo	521.7±5.3*	10^1	526.9±50.6*	0.99±0.2*
		10^2	1304±145*	0.4±0.1*
		10^3	-	-

* – statistically significant differences between histochrome and other drugs ($p \leq 0,05$).

For example, histochrome inhibited viral replication at an infectious dose of TBEV 10^2 $TCID_{50}$/ml at an IC_{50} concentration of 21.8±2.6 μg/ml, while its SI was 2.5±0.2. The indices for the infectious dose of TBEV 10^2 $TCID_{50}$/ml in oxolin: IC_{50} = 95.1±10.0 μg/ml and SI = 1.1±0.1, and placebo IC_{50} = 1304±145 μg/ml and SI = 0.4±0.1.

Antiviral activity of the tested preparations against herpes simplex virus type 1 (HSV-1).

Based on the results of the MTT assay, 50% cytotoxic concentration (CC_{50}) and the main antiviral activity against various infectious doses of the herpes simplex virus type 1 as described above were calculated for each preparation. The results of the study are presented in Table 2.

As can be seen from Table 2, histochrome is much more effective in suppressing the replication of HSV-1 than the placebo preparation, an antioxidant composition containing ascorbic acid and α-tocopherol. Histochrome possesses a higher toxicity (CC_{50} = 60.5±3.1 μg/ml) and suppresses the virus at lower concentrations than placebo (CC_{50} = 530.9±9.4 μg/ml), and accordingly has a higher therapeutic index (SI).

Table 2

Antiviral activity of the tested preparations against herpes simplex virus type 1 (HSV-1)

Preparation	Cytotoxicity CC_{50}, μg/ml	Infectious dose ($TCID_{50}$/ml)	Inhibiting concentration IC_{50}, μg/ml	Selectivity index (SI)
Histochrome	60.5±3.1	10^1	8.9±0.9	6.8±0.6
		10^2	18.8±2.1	3.2±0.3
		10^3	40.3±4.4	1.5±0.1
Placebo	530.9±9.4*	10^1	482.5±53.1*	1.1±0.1*
		10^2	885±97*	0.6±0.1*
		10^3	-	-

* – statistically significant differences between histochrome and other drugs (p≤0,05).

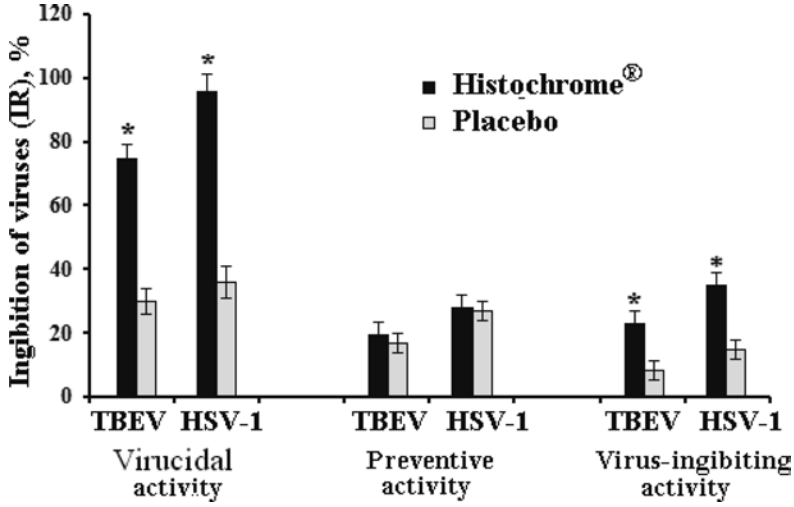

Fig. 1. Virucidal, preventive and virus-inhibiting activity of the Histochrome® drug.
Note: TBEV is a tick-borne encephalitis virus, HSV-1 is a herpes simplex virus type 1, * - statistically significant differences between the values of histochrome and placebo (p≤0.05)

If the infectious dose of HSV-1 was 10^2 $TCID_{50}$/ml, the IC_{50} histochrome index = 18.8±2.1 μg/ml, and SI = 3.2±0.3, which was significantly higher than in the placebo – IC_{50} = 885±97 μg/ml and SI = 0.6±0.1.

The comparative antiviral efficacy of histochrome, oxolin and placebo preparations was determined at an infectious dose of TBEV and HSV-1 of 10^2 $TCID_{50}$/ml and the concentration of preparations 20 μg/ml at different stages of the viruses' life cycle. The virucidal effect was investigated – the effect of the preparations on the viruses themselves, the prophylactic action – the effect of the preparations on the cells pretreated with the preparations before infection with viruses, and the virus-inhibiting effect – the effectiveness of the preparations in the early stage of viral replication. The antiviral activity of the preparations was assessed by the degree of inhibition of the cytopathic effect of the viruses by the MTT assay as described above.

Figure 1 shows the virucidal, preventive and virus-inhibiting activity of preparations against TBEV and HSV-1.

CONCLUSION

It has been found that when the virus is pretreated with histochrome preparation (virucidal activity) the maximum inhibition of TBEV and HSV-1 is 78% and 96%, respectively, and 30% when applied to placebo. The degree of inhibition of viruses in the preventive use of both histochrome and placebo against TBEV and HSV-1 was 20% and 30%. When TBEV and HSV-1 were treated with histochrome at the early stage of replication, the virus-inhibiting effect was 25 and 38%, respectively, and for placebo, 5% and 15%.

Thus, it was shown for the first time that histochrome exhibits antiviral activity against tick-borne encephalitis and herpes simplex type 1 and acts at the early stages of the life cycle of these viruses.

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