One of the problems encountered in the chemotherapy of viral infections is the formation of mutant virus variants that are resistant to drugs. The probability of drug resistance development directly depends on the efficiency of viral replication suppression in the course of chemotherapy. If viral replication in the infection focus is inhibited partly, the conditions for selection of resistant variants of the virus existing in the virus population are created [1–3]. The use of binary and ternary combinations of drugs with different mechanism of antiviral action can significantly improve their antiviral activity, thereby ensuring the maximum therapeutic effect even when drugs are used at concentrations lower than the concentration required for monotherapy. This reduces the risk of development of virus-resistant populations or even prevents their occurrence, which was shown both in vitro experiments and in clinical conditions using HIV-1 and hepatitis C models [4, 5], as well as a model of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) [1, 6, 7].

Previously we found that the use of ACV in combination with dimeric netropsin analogues had a synergistic effect in suppressing the HSV-1 reproduction in Vero E6 cells [7]. The observed effect is based on the difference in the mechanisms of action of ACV and dimeric netropsin analogues on the replication of viral DNA: ACV inhibits the stage of elongation during the synthesis of DNA by viral DNA polymerase, whereas bis-netropsins inhibit the process of initiation during viral DNA replication [10–12]. However, the effect of the combined use of these compounds on the rate of formation of an HSV-1 population resistant to one of these drugs or their combination has been studied insufficiently. This fact served as a basis for the study, the results of which are presented in this paper.

Previously, we found that the dimeric derivatives of antibiotic netropsin selectively bind to the A+T cluster at the replication origin of herpes simplex virus (OriS and OriL) and effectively suppress viral replication in Vero E6 cells [10, 11]. When bound to the A+T cluster in OriS, bis-netropsins increase the melting temperature of the A+T cluster and reduce the probability of the “opening” of AT base pairs caused by thermal fluctuations [10–12], which is required for the initiation of DNA unwinding by helicase UL9 of virus herpes. Unlike ACV, bis-netropsins inhibit the initiation of viral DNA replication, i.e. function at the early stages of the virus life cycle before the viral DNA synthesis begins.

Figure 1 shows the chemical formulas of bisternetropsins, whose antiviral activity in Vero E6 cells as well as their cytotoxicity was studied by us earlier [1, 7, 11]. It was shown that Pt-bis-Nt and 15Lys-bis-Nt effectively inhibit the reproduction of both ACCV-sensitive and ACV-resistant HSV-1 variants, whereas Pt*-bis-Nt was ineffective. Figure 2 shows the melting curves of the free 63-mer oligonucleotide S1, present at the beginning of OriS replication of herpes virus and its complexes with 15Lys-bis-Nt in a 1 : 1 ratio and Pt*-bis-Nt. In solution, the oligonucleotide S1 spontane-
ously folds into two hairpins—a GC-rich hairpin, which selectively bound to UL9 helicase herpes virus, and an AT-rich hairpin, which binds to bis-netropsin. Figure 2 shows that the shape of the melting curves of the complexes of 15Lys-bis-Nt and Pt*-bis-Nt with the AT-rich hairpin in the oligonucleotide S1 vary considerably. 15Lys-bis-Nt increased the melting temperature of the AT-rich hairpin by 24°C, whereas Pt*-bis-Nt increased the melting temperature of the hairpin by only 10°C.

**Fig. 1.** Chemical structure of the antibiotic netropsin (Nt) and its dimeric analogues Pt-bis-Nt, Pt*-bis-Nt and 15Lys-bis-Nt.
The purpose of this work was to study the antiviral activity of the dimeric netropsin derivative 15Lys-bis-Nt used in combination with acyclovir and to investigate the drug resistance development. 15Lys-bis-Nt was synthesized at the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences (RF patent No. RUS 2265610 20.04, 2004), and its DNA-binding and antiviral activity were studied in [7, 11, 12]. Currently, 15Lys-bis-Nt is at the preclinical trial stage.

Experiments were performed with Vero E6 cells derived from green monkey kidney. Herpes simplex virus type 1 strain L2 (HSV-1/L2) was obtained from the National Collection of Viruses, Ivanovsky Research Institute of Virology, Russian Academy of Medical Sciences.

**Virus passage conditions.** In this study, we used a multiplicity of infection (MOI) of 1 PFU/cell, which allowed us to increase the number of ACV-resistant virus particles present in the original virus population as minor components.

**Virus sensitivity** to the test compounds was studied in accordance with the internationally accepted method of inhibiting the development of virus-induced cytopathic effect (CPE) as was described in detail previously [10, 11]. Virus sensitivity was expressed as IC50—the concentration of the compound at which a 50% inhibition of viral CPE was observed compared to the control (MOI 0.1 PFU/cell, incubation duration 48 h).

**The combined effect** of the compounds was evaluated by constructing an isobologram [14].

In accordance with international guidelines on dosing ACV for the treatment and prevention of recurrences of genital herpes, ACV is given per os at a dose of 400 mg 3–5 times per day, which corresponds to the ACV concentration in the blood plasma of 0.94–1.97 $\mu$g/mL [15]. To obtain a resistant population, the concentration of ACV in the maintenance medium during the serial passaging of the virus in the cell culture was 1.50 $\mu$g/mL, which corresponds to approximately $4 \times IC_{50}$.

When studying the effect of 15Lys-bis-Nt on the rate of development of HSV resistance to ACV, the passage conditions were comparable to those used for ACV alone. For this purpose, the concentration of 15Lys-bis-Nt in combination with ACV was selected in such a way that their combined effect was also equal to $4 \times IC_{50}$. To determine the concentrations of 15Lys-bis-Nt and ACV that, in the case of the combined use of these compounds, provides 50% inhibition of viral CPE compared to the control (which corresponded to IC50), we studied the combined effect of 15Lys-bis-Nt and ACV, which was evaluated by constructing an isobologram as shown in Fig. 3. These experiments confirmed the previously established well-expressed synergistic interaction of these compounds in the case of their combined use [7, 10]. For example, under the combined influence of 15Lys-bis-Nt and ACV, 50% inhibition of virus-induced CPE can be reached at

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**Fig. 2.** Melting curves of the complexes of dimeric netropsin analogues Pt*-bis-Nt and 15Lys-bis-Nt with the 63-mer oligonucleotide S1, contained in the replication origin OriS of herpes simplex virus. The melting temperatures (Tm) of the AT-rich hairpin in the free oligonucleotide S1 and the complexes formed by the oligonucleotide with bis-netropsins are shown. The melting curves were obtained by FRET (Forster resonance energy transfer). The dye carboxyrhodamine 6G and the quencher Black Hole 2 were covalently linked to the 5' and 3' ends of the oligonucleotide S1. The ordinate axis shows the fluorescence intensity of the fluorophore at 557 nm. Conditions: 0.01 M cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl.
concentrations 4 times smaller than in the case of individual use of these drugs.

Based on the data presented in Fig. 3, we have selected two combinations of concentrations of the test compounds corresponding to IC50, at which they were used in different proportions. In the first combination, the concentrations of 15Lys-bis-Nt and ACV were 0.1 + 0.2 µg/mL, respectively (ratio, 1 : 2); in the second, 1.5 + 0.05 mg/mL, respectively (30 : 1). For these mixtures, the concentrations corresponding to 4 × IC50 were used for HSV passaging. These concentrations were equal to (0.4 + 0.8) µg/mL and (6.0 + 0.2) µg/mL, respectively.

To assess the decrease in the sensitivity of the virus population during passaging in the presence of the test compounds alone or in combination, we determined IC50 values for ACV and 15Lys-bis-Nt in the material of each passage. As can be seen from the data presented in Fig. 4, when HSV-1 was passaged in the presence of ACV alone, an ACV-resistant virus population was rapidly formed (curve 1). The sensitivity of material of the second passage was reduced 16 times compared to the baseline sensitivity. The material of the third passage was already highly resistant to ACV: IC50 value for ACV was 32 times higher than the baseline value.

When HSV-1 was passaged in the presence of a combination of 15Lys-bis-Nt and ACV, a gradual decrease in the sensitivity of the virus population to ACV was observed. However, the rate of formation of the resistant population was lower than during the passaging of the virus in the presence of ACV alone. When the ratio of 15Lys-bis-Nt and ACV was 1 : 2 (Fig. 4, curve 2), the population whose sensitivity was reduced 16 times was obtained after three successive passages. The material of the fourth and fifth passages was 32 times less sensitive to ACV. When the proportion of 15Lys-bis-Nt in the combination with ACV was increased (ratio 30 : 1, curve 3), a viral population with a 16-fold lower sensitivity to ACV was obtained; however, this decrease required five passages. After two more passages, the sensitivity decreased 32 times.

Thus, it can be concluded that there is a relationship between the rate of formation of a resistant viral population and the weight ratio of the combined compounds: the smaller the proportion of ACV in the mixture, the slower the decrease in the virus population sensitivity to ACV during passaging.

The experimental conditions used in the study allowed us to investigate the possibility of obtaining an HSV-1 population resistant to 15Lys-bis-Nt or a population resistant simultaneously to 15Lys-bis-Nt and ACV. Fifteen serial passages in the presence of 15Lys-bis-Nt alone at a concentration of 4 × IC50 (12.5 µg/mL) did not affect the viral sensitivity to 15Lys-bis-Nt (Fig. 4, curve 4).

As we have shown previously, the mechanism of the antiviral action of 15Lys-bis-Nt was associated with the inhibition of the helicase activity of the viral protein UL9 [12]. This protein binds selectively to the conserved sequences (boxes) flanking A+T clusters in three sites of viral DNA corresponding to three DNA replication origins (two OriS sites and one OriL site in the small and large fragments of viral DNA, respectively). Upon binding to the A+T cluster, 15Lys-bis-Nt prevents the fluctuation opening of AT pairs, which is required for the initiation of DNA unwinding by the helicase UL9. The antiviral activity of 15Lys-bis-Nt is determined by the interaction of this compound with
an extended region (14–16 AT base pairs) in the A+T cluster at the DNA replication origin OriS or OriL. The binding constant of bis-netropsin in this region of viral DNA depends on the width of the narrow DNA groove and the nucleotide sequences in the binding site. Multiple mutations that theoretically could cause resistance to 15Lys-bis-Nt should be located in the binding site for bis-netropsin in the replication origin of viral DNA. After 15 serial passages of HSV-1 in the presence of 15Lys-bis-Nt, mutant virus variants with a lower sensitivity to 15Lys-bis-Nt compared to the standard virus variant were not found. It can be assumed that, to obtain virus variants resistant to 15Lys-bis-Nt, it is necessary to introduce dramatically greater changes into the virus genome than is the case of formation of resistance to ACV. It is of interest to continue searching the herpes virus variants resistant to 15Lys-bis-Nt and other dimeric netropsin analogues. A herpes virus variant with a much lower sensitivity to the antibiotic distamycin than the standard virus variant was described in [13]. However, the molecular mechanisms that are responsible for the observed differences in the sensitivity of different herpes virus variants to distamycin are not known. When the virus was passaged in the presence of a combination of 15Lys-bis-Nt and ACV, only the sensitivity of the virus to ACV was reduced, whereas the sensitivity to 15Lys-bis-Nt remained unchanged.

The results of this study led us to conclude that, unlike the use of ACV and other modified nucleosides, the use of 15Lys-bis-Nt did not yield a drug-resistant HSV-1 variant. The use of ACV in combination with 15Lys-bis-Nt does not prevent the development of an ACV-resistant HSV-1 population; however, the process of resistance development in this case is significantly decelerated.

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REFERENCES


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