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ONCOLYTIC POTENTIAL OF FISH RHABDOVIRUS

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Aim. The goal of the work was to study the effect of fish rhabdovirus (Spring viraemia of carp virus — SVCV) on cultured human cervical carcinoma (HeLa) cells. This virus is not pathogenic for humans and accumulates in large quantities *in vitro*, so it is of considerable interest to investigate the possibility of its use as an oncolytic agent.

Methods. For cultivation of fish rhabdovsrus (SVCV) the susceptible fish cell line EPC were used in culture assay. The virus was purified from tissue culture supernatant by the method of ultra-centrifugation. Human cervical carcinoma cells of the HeLa line (ECACC catalog no.93021013) were cultured in RPMI-1640 medium (Sigma, USA). Determination of the cytotoxic/cytostatic effect on cells exposed to SVCV was carried out using the MTT test. To determine the distribution of cells by cycle phases under the influence of SVCV and in control cell samples, the method of flow cytofluorimetry was used.

Results. In this work, it is shown for the first time that a fish rhabdovirus significantly inhibits the growth of HeLa cells derived from human cervical carcinoma and causes the appearance of apoptotic bodies in them. Determination of the cytotoxic/cytostatic effect of SVCV, carried out in successively decreasing dilutions of the culture medium, showed that it significantly inhibits the growth of HeLa cells compared to the control at dilutions of 1:2, 1:4 and 1:8.

Conclusions. As the results of our research, it was shown fish rhabdovirus (SVCV) could be included in the extensive list of oncolytic rhabdoviruses. It significantly inhibits the growth of HeLa cells derived from human cervical carcinoma and causes the appearance of apoptotic bodies in them. Further, *in vivo* studies are needed for a comprehensive evaluation of its oncolytic properties.

Key words: Fish rhabdovirus, SVCV, oncolysis, cell cultures, apoptosis.

More than 40 viruses from 10 different families have oncolytic properties, many of which are undergoing clinical trials (phases I, II, III), and some have already been approved for use [1]. The first oncolytic virus, a picornavirus called RIGVIR, was approved in Latvia for the treatment of melanoma in 1989, but, apart from Georgia, Armenia and Uzbekistan, it has not been widely used [2]. Since 2001, the oncolytic drug DELYTACT, based on a modified herpes virus, has been approved in Japan for the treatment of malignant gliomas [3], and in China, in 2005, the drug ONCORINE [4], based on a genetically modified adenovirus Ad (H101), was approved for the treatment of nasopharyngeal carcinoma. In 2015, the oncolytic drug TALIMOGENE LAHERPAREPVEC (T-VEC), which was created using genetic engineering based on the herpes virus (HSV-1), into the genome of which the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene was

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integrated to enhance the immune response, was approved in the USA for the treatment of postoperative melanoma recurrences [5]. In addition to the USA, T-VEC is currently approved for use in Israel, Australia, and Europe [6]. In recent years, animal rhabdoviruses have found wide application in various fields of biotechnology, including for the treatment of various forms of cancer [7–9].

Materials and Methods

Cell cultures. Spring viremia of carp virus (isolate Svirneve VN17) was isolated from carp *Cyprinus carpio* [10]. For virus propagation the cell line EPC [Epithelioma Papulosum Cyprini] were used. EPC cells were maintained in RPMI 1640 medium (Euroclone, Italy) supplemented with 1% antibiotic-antimycotic solution and 10% foetal bovine serum (Sigma, USA). The virus suspension was inoculated onto 24-hours cell monolayer growing in 25 cm^2 flasks. After absorption for 60 min at 15 °C, RPMI 1640 medium supplemented with 2% of FBS was added to cells. When a complete viral cytopathic effect (CPE) was evident, tissue culture supernatant was harvested and centrifuged at 2500 × g for 10 min at 4 °C to remove cell debris. The 50% tissue culture infective dose (TCID50 ml^{-1}) of the resulting supernatant was determined.

Human cervical carcinoma cells of the HeLa line (ECACC catalog no.93021013) were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% FBS (Sigma, USA), 2% glutamine, and 1% antibiotic-antimycotic (Thermo Fisher Scientific, USA) at 37 °C in a 100% humidified atmosphere and 5% CO_2.

Virus purification. The virus was purified from tissue culture supernatant by the method of ultracentrifugation. Briefly, after cell debris was separated by centrifugation at 2500× g for 10 min at 4 °C, the pellet was discarded, and the supernatant was centrifuged in Beckman L5-50B in a rotor of SW-40 for 60 min at 70500 × g at 4 °C. The virus pellet was suspended in TNE (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) and centrifuged at 2500 × g for 5 min at 4 °C.

Cytotoxic/cytostatic effect of SVCV. The study of the cytotoxic/cytostatic effect of SVCV in the dilution range of 1:128 — 1:2 was carried out on the HeLa cell line. Determination of the cytotoxic/cytostatic effect on cells exposed to SVCV was carried out using the MTT test. For this purpose, cells were plated in RPMI 1640 media (Sigma, USA) containing 10% fetal calf serum and 1% ng/ml of an antibiotic/antimycotic mixture at a concentration of 1×10^5 cells/ml in a volume of 100 µl at a concentration of 1×10^5 cells/ml. After the cells reached 60–70% monolayer, SVCV was added in serially decreasing dilutions of 1:128–1:2 to the wells of a 96-well plate with cells and incubated for 48 hours. The control wells contained medium without virus (control). Four hours before the end of the incubation period, 20 µl of MTT reagent (colorless tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., USA)) was added to a final concentration of 0.6 mM and incubated in a CO_2 incubator under standard conditions for the cells to reduce soluble MTT reagent to insoluble formazan crystals. The plate was gently shaken for 5–10 min to dissolve the formazan crystals, and the optical density of each well was determined at 540 nm (using a BioTek multiwell spectrophotometer, USA) by subtracting the measured background absorbance at 620 nm. The intensity of the staining correlated with the number of living cells in the incubation medium. Statistically processed results were presented as $M \pm m$.

Flow Cytometry. To determine the distribution of cells by cycle phases under the influence of SVCV in a dilution of 1:8 and in control cell samples, the method of flow cytofluorimetry was used. For the preparation of one sample, no less than 5×10^5 cells were used. The cells were pelleted by centrifugation at 1000 g for 5 min. The supernatant was collected, and the cells were washed with physiological or phosphate-buffered saline (PBS, pH 7.2). The cells were resuspended in 200 µl of PBS, 300 µl of citrate buffer (pH 6.8) containing 0.1% Triton X-100 was added. After 1 minute, 10 µl of ribonuclease and 10 µl of propidium iodide (Sigma, USA) were added to stain DNA for 5 min. Incubated for 10 min at 37 °C in the dark and 30 min at room temperature. Centrifuged at 1000 g for 10 min and removed the supernatant. After that, the cells were fixed by adding 400 µl of PBS, with 0.4% formalin and analyzed for DNA content in the samples. Measurements were performed using a flow cytometer. To determine the number of cells in different phases of the cell cycle, cytofluorimetric analysis was performed using an argon laser-equipped flow cytometer (Becton Dickinson, USA) (excitation $\lambda = 488$ nm, emission $\lambda = 585/40$ nm). Samples were analyzed using the Mod Fit LT 3.0 program (BDIS, USA).

Results and Discussion

Spring viraemia of carp (SVC) is a rhabdovirus infection capable of inducing an acute haemorrhagic and contagious viraemia in several carp species and of some other cyprinid and ictalurid fish species. SVCV (isolate Svirneve VN17) was isolated in Ukraine from yearlings of the common carp *Cyprinus carpio* [10]. For virus isolation and characterization, methods of cell culture, EM, PCR, and sequencing were used. Preliminary examination of infected fish revealed a range of lesions, particularly in spleen and kidney tissues. The phylogenetic analysis revealed a close relationship of Ukrainian isolates of SVCV with sequences that represent the strains from genogroup Ib [10].

In EPC-infected cells CPE occured at 48 h after infection. The virus grew in fish cell line EPC with infectious titre of $10^{6.9–7.5}$ $TCID_{50}$/ml. The infected cells were rounded, then detached from the substrate and lysed. Among the SVCV strains isolated by us in different regions of Ukraine, the highest infectious titers in EPC cells (in range of $10^{6.9–7.5}$ $TCID_{50}/ml^{-1}$) were the isolates from Lviv (VN2), Donetsk (VN4) and Cherkasy regions (VN17).

The reproduction of SVCV (isolate Svirneve VN17) in HeLa cells was accompanied by significant changes in the cell culture. Determination of the cytotoxic/cytostatic effect of SVCV, carried out in successively decreasing dilutions of the culture medium, showed that it significantly inhibits the growth of HeLa cells compared to the control at dilutions of 1:2, 1:4 and 1:8 (Fig. 1).

To determine possible mechanisms of action, the effect of SVCV (isolate Svirneve VN17) in a 1:8 dilution on the level of apoptosis and the content of cells in different phases of the cell cycle was evaluated. As shown by the results, the level of apoptotic cells increased by twofold compared to the control (Fig. 2).

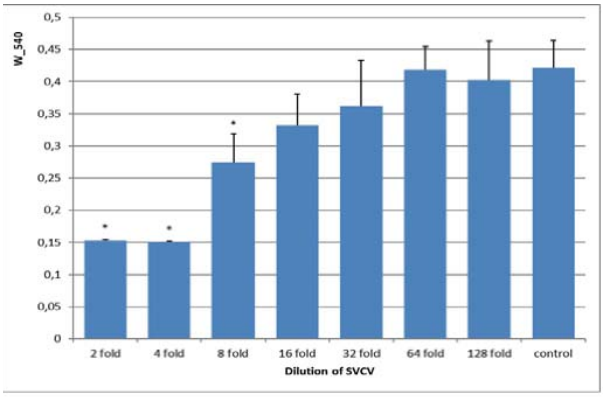

Fig. 1. **Effect of SVCV (isolate Svirneve VN17) on cultured HeLa cells (human cervical cancer) at different dilutions. MTT assay data**

The content of cells in the proliferative pool (S+G2/M) was especially significantly reduced. Thus, the ratio of G1, G2 and S phases in HeLa cells for 48 hours incubation period with the inoculation of SVCV was G1 — 61.45%, G2 — 26.35% and S — 12.20%. In the control, the ratio of phases was as follows — 59.87%, G2 — 9.64% and S — 30.49%, respectively (table 1). As can be seen from the results, there is no difference between the experimental and control variants in the G1 phase. The evident effect of the virus on the cells was in the S phase by almost three times reduction (Fig. 3). It should also be noted that cells quantity in the G2 phase increased due to virus action, and presumably, the synthesis of viral proteins increased. The content of cells in the proliferative pool (S+G2/M) in control variant is shown in Figure 3.

The possibility of creating oncolytic drugs based on animal rhabdoviruses, especially those that are not pathogenic to humans, has attracted much attention from researchers in recent years. The first report on the ability of rhabdoviruses to inhibit the development of tumors was published in Italy at the beginning of the 20th century. De Pace [11] observed tumor regression after inoculation of an attenuated rabies vaccine in a patient with cervical cancer. Another patient with metastatic melanoma who was vaccinated against rabies after a dog bite also had a prolonged remission [12]. Among animal rhabdoviruses, the largest number of works on oncolysis was performed on the model of representatives of the genus *Vesiculovirus*, such as VSV, Maraba, Morreton, and Jurona.

VSV has been shown to be highly effective against malignant glioma, melanoma, hepatocellular carcinoma, breast adenocarcinoma, certain leukemias, and prostate tumors [8, 13, 14, 15, 16]. In the laboratory of Prof. Spivak M.Ya. (D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine), the ability of VSV to suppress the development of experimentally induced tumors in the Kalanchoe plant was also discovered. When incubating plant tumors induced by the bacterium *Agrobacterium tumefaciens* with the vesicular stomatitis virus, tumor regression by 74.5% was noted. Under the influence of buckwheat burn rhabdovirus,

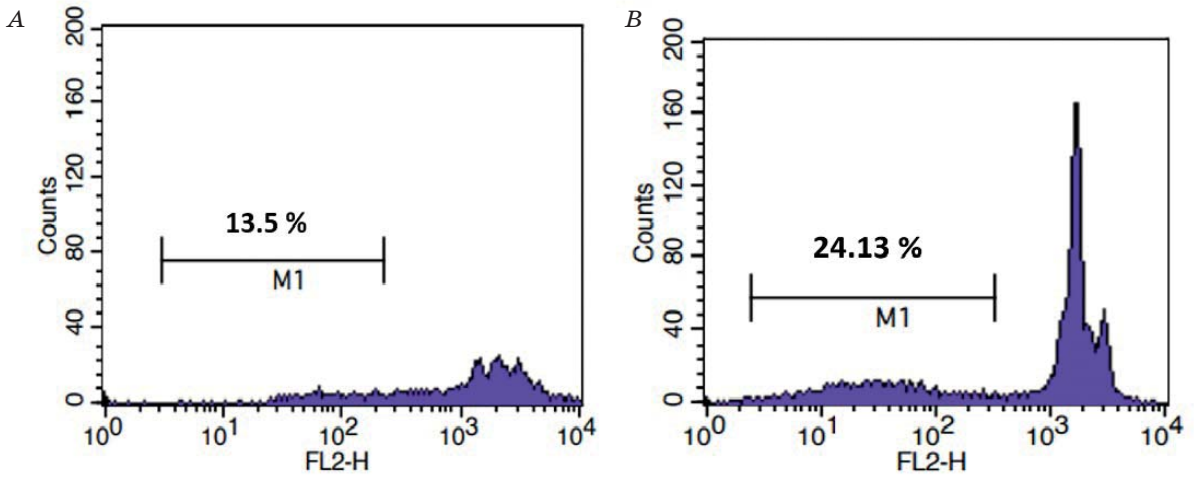

Fig. 2. **The effect of SVCV (isolate Svirneve VN17) at a dilution of 1:8 on the apoptotic cell amount:** *A* — HeLa cells without treatment; *B* —HeLa cells treated with SVCV at 1:8 dilution (incubation for 2 days in standard CO_2 incubator conditions at 37 °C, 100% humidity in RPMI-1640 medium with 10% FBS)

Table 1

HeLa proliferative activity under SVCV (isolate Svirneve VN17) treatment

Groups	Cell cycle phases, %		
	G1	S	G2+M
Control	59.87±2.9	30.49±1.5	9.64±0.5
SVCV treatment	61.45±3.1	12.20±0.6	26.35±1.3

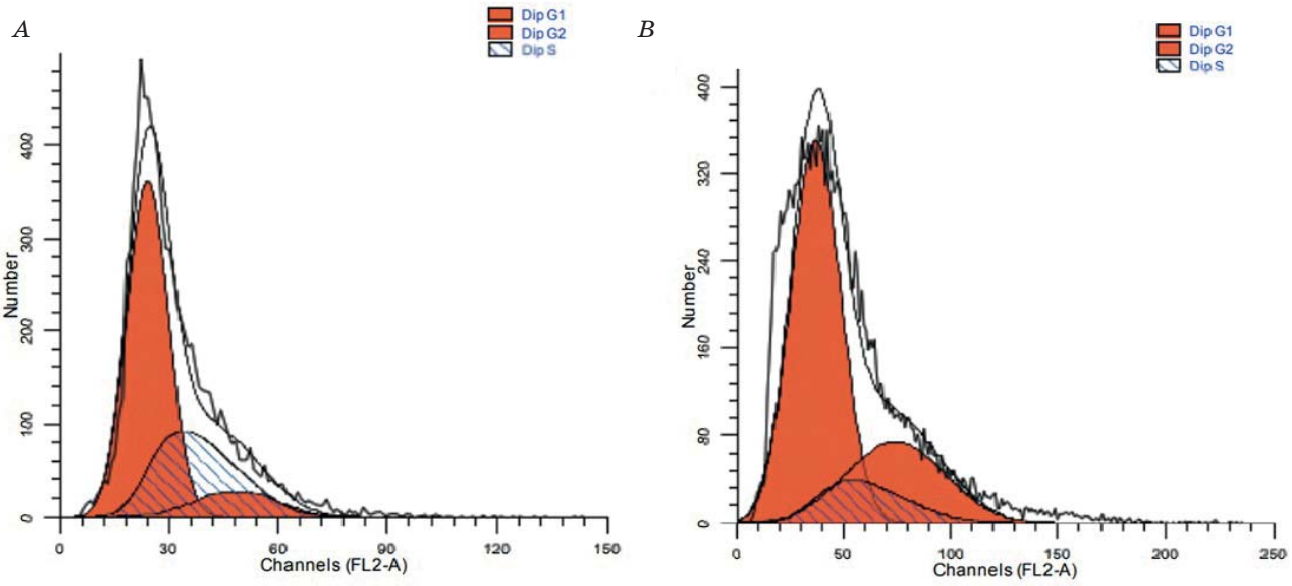

Fig. 3. **The cell cycle distribution of HeLa cells treated with SVCV(isolate Svirneve VN17):** *A* — HeLa cells without treatment; *B* — HeLa cells treated with SVCV at 1:8 dilution (incubation for 2 days in standard CO2 incubator conditions at 37 °C 100% humidity in RPMI-1640 medium 10% FBS)

apoptotic bodies appeared in 90% of Sarcoma 37 cells [17]. Other animal rhabdoviruses, such as Bahia Grande (BGV) and Muir Springs (MSV) baraviruses, also have oncolytic properties [18].

Conclusions

As the results of our research, it was shown fish rhabdovirus (SVCV) could be included in the extensive list of oncolytic rhabdoviruses. It significantly inhibits the growth of HeLa cells derived from human cervical carcinoma and causes the appearance of apoptotic bodies in them. This virus can serve as a basis for creating an oncolytic drug to combat cancer.

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Conflicts of Interest

The authors declare no conflicts of interest. This article does not contain any human or animal studies conducted by any of the authors.

Author Contributions

L. Buchatskyi — conceptualized the study, literature review, editing; Yu. Rud — virus isolation, virus cultivation and purification, data analysis; L. Garmanchuk — HeLa cells cultivation, cytotoxic/cytostatic effect of SVCV, paper writing, figure preparation; O. Gorbach — data analysis.

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ОНКОЛІТИЧНИЙ ПОТЕНЦІАЛ РАБДОВІРУСУ РИБ

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Мета. Метою роботи було вивчення впливу рабдовірусу весняної віремії коропа (SVCV) на культивовані клітини раку шийки матки людини (HeLa). Вірус весняної віремії коропа не є патогенним для людини та накопичується у великих кількостях *in vitro*, тому представляє значний інтерес при дослідженні його можливості у використанні як онколітичного засобу.

Методи. Для культивування рабдоврусу риб (SVCV) усі зібрані в рибницьких господарствах України зразки були протестовані за допомогою клітинного культурального аналізу. Вірус був очищений з супернатанту тканинної культури методом ультрацентрифугування. Клітини карциноми шийки матки людини лінії HeLa культивували у середовищі RPMI-1640 (Sigma, США). Визначення цитотоксичного/цитостатичного ефекту на клітини, що зазнали впливу SVCV, проводили за допомогою МТТ-тесту. Для визначення розподілу клітин за фазами циклу під впливом SVCV та у контрольних зразках клітин використовували метод проточної цитофлуориметрії.

Результати. Вперше показано, що рабдовірус риб (вірус весняної віремії коропа — SVCV) значно пригнічує ріст клітин HeLa, отриманих з раку шийки матки людини, та викликає появу в них апоптотичних тілець. Визначення цитотоксичної/цитостатичної дії SVCV, проведене в послідовно зменшуваних розведеннях культурального середовища, показало, що він значно пригнічує ріст клітин HeLa порівняно з контролем при розведеннях 1:2, 1:4 та 1:8.

Висновки. Результати дослідження показали, що рабдовірус риб (SVCV) можна включити до широкого переліку онколітичних рабдовірусів. Він значно пригнічує ріст клітин HeLa, що походять від раку шийки матки людини, та спричиняє появу в них апоптотичних тілець. Необхідні подальші дослідження *in vivo* для комплексної оцінки його онколітичних властивостей.

Ключові слова: рабдовірус риб, SVCV, онколізис, культура клітин, апоптоз.