Frontiers in Bioscience Landmark

*Front. Biosci. (Landmark Ed)* **2024**; 29(11): 374
https://doi.org/10.31083/j.fbl2911374

*Review*

# Oncolytic Vesicular Stomatitis Virus: Optimisation Strategies for Anti-Cancer Therapies

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Academic Editor: Roberto Bei
Submitted: 1 June 2024 Revised: 22 July 2024 Accepted: 31 July 2024 Published: 31 October 2024

**Abstract**

Oncolytic viruses (OVs) represent a targeted anti-cancer therapy approach due to their ability not only to selectively infect and destroy malignant cells but also to induce an immune response. Vesicular stomatitis virus (VSV) offers a promising platform due to its low prevalence and pathogenicity in humans, lack of pre-existing immunity, easily manipulated genome, rapid growth to high titers in a broad range of cell lines, and inability to integrate into the host genome. However, despite its many advantages, many unresolved problems remain: problematic production based on the reverse genetics system, oncological selectivity, and the overall effectiveness of VSV monotherapy. This review will discuss various attempts at viral genome modifications aimed at improving the oncolytic properties of VSV. These strategies include inhibition of viral genes, modification of genes responsible for targeting cancer cells over healthy ones, insertion of foreign genes for boosting immune response, and changing the order of viral and inserted foreign genes. In addition, possible ways to improve VSV-based anti-tumor therapy and achieve higher efficiency will be considered by evaluating the effectiveness of various delivery methods as well as discussing treatment options by combining VSV with other groups of anticancer drugs.

**Keywords:** oncolytic virus; vesicular stomatitis virus; immunotherapy; anti-cancer therapy

## 1. Introduction

Cancer is a disease in which mutated cells grow and divide uncontrollably, a process that leads to malignant tumor growth. These cells are able to spread to nearby tissues and organs and further throughout the body with the formation of metastasis [1]. In 2020, the Global Cancer Observatory (GCO) reported approximately 19.3 million new cancer cases and almost 10 million cancer deaths [2]. Preliminary forecasts predict an increase in these figures over time [3]. While various types of cancer treatment, such as surgery, chemotherapy, radiation therapy, hormone therapy, and immunotherapy, have existed for many years [4], they have not always led to successful outcomes for patients.

Oncolytic viruses (OVs) are able to selectively replicate in malignant cells, deliver immunostimulatory factors, and activate the host immune response [5]. While only several OV-based therapies have been registered in the world so far (Rigvir™, Oncorine™, Imlygic™, Adstiladrin™ and Delytact™), the number of OV-based clinical trials is steadily growing and currently exceeds two hundred (www.clinicaltrials.gov) [6,7]. Oncorine™, a recombinant human adenovirus type 5 (AdV5)-based therapy for the treatment of late-stage refractory nasopharyngeal cancer, was approved in China in 2005 [8]. Rigvir™ (ECHO-7), an echovirus-based therapy approved in Latvia in 2004 for the treatment of melanoma, has since been removed from this list. Imlygic™, a modified herpes simplex virus type 1 (HSV-1) delivering human granulocyte-macrophage colony-stimulating factor (GM-CSF) for the treatment of melanoma, was approved in 2005 in China and in 2015 in the USA [9]. Delytact™, a recombinant HSV-1 for the treatment of glioblastoma, was registered in 2021 [10]. Adstiladrin™, a non-replicating oncolytic adenovirus delivering interferon (IFN) alfa-2b for the treatment of bladder cancer, was approved in the USA in 2022 [11].

A number of viruses, such as HSV, AdV, Newcastle disease virus (NDV), and reovirus, possess oncolytic abilities and, therefore, can potentially be potent OVs [12,13]. The absence of neutralizing antibodies (nAb) in humans, present against HSV, gives a great advantage to vesicular stomatitis virus (VSV) when developing oncolytic therapy, yet HSV-based OV therapy is currently under intense investigation. It is worth noting that VSV production is not as simple [14] as that for AdV, for instance, and large gene insertion may restrict the speed of VSV replication [15]. Despite this fact, VSV continues to be a promising OV. VSV is a prototypic non-segmented negative-strand RNA virus belonging to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Vesiculovirus*. It should be noted that morphologically and genetically related viruses belong to the genus *Vesiculovirus*, which is why the concept of 'VSV' covers a number of viruses that belong to the same group. This group consists of Vesicular stomatitis New Jersey virus

(VSNJV), Vesicular stomatitis Indiana virus (VSIV), Cocal virus (COCV), Vesicular stomatitis Alagoas virus (VSAV), and Morreton virus (MORV). However, the type species is still considered to be VSIV [16].

The relatively small 11 kb VSV genome encodes five proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L) [17,18], separated by a section of two C/GA nucleotides [18,19]. The 47-nt-long leader sequence (Le) at the 3′ end of the genome is responsible for binding RNA-dependent RNA polymerase (RdRp), replication, and transcription of the genome [20,21]. The 54-nt-long 5′-terminal sequence (Tr) contains sequences necessary for replication and packaging of the newly synthesized RNAs into particles [21,22]. Similar to other non-segmented RNA viruses, VSV transcription begins with the promoter of the 3′ terminus-proximal N gene, and monocistronic mRNA is generated with sequential transcription of each gene, which is a prerequisite for transcription [23]. Therefore, the proximal gene is transcribed in the largest amount, and the amount of each following gene drops by about 25–30% [19]. This process is called attenuation and occurs due to RdRp dissociation during transcription [24].

Each VSV protein has a unique and important function for viral replication (Fig. 1). The entire viral genome is encapsulated by N to form a ribonucleoprotein complex (RNP) [25]. The G on the surface of the virus is responsible for penetration into the cell *via* the attachment of low-density lipoproteins (LDLR). This transmembrane protein is involved in the uptake of cholesterol molecules bound to lipoproteins, and this may explain the wide viral tropism of VSV [26]. Cell entry occurs as a result of actin-dependent clathrin-mediated endocytosis after the virus-host cell membrane fusion [27,28]. The RdRp, responsible for transcription and replication, is contained within L associated with RNP through P [29]. The RNP, after interaction with matrix protein, condenses into a typical spiral structure with G on the surface [30,31]. The switching of the RdRp complex from genome transcription to protein translation is activated by the accumulation of viral proteins, with N playing the most important role in this process [32]. M participates in virus assembly, inhibits host cell gene expression [33], and also protects the virus itself from the innate immune reactions of the host organism [34,35].

VSV infects various animals, such as horses, cattle, and pigs [32] and also causes rare sporadic outbreaks [36]. At the same time, infection with this virus is not fatal for animals and is characterized by the appearance of blisters in the oral cavity, areas of the paws, and nipples [36]. However, the most interesting aspect from the point of view of both molecular biology and research is that this virus is an insignificant pathogen for humans since infection is asymptomatic, and its genome can be easily modified, making it a popular platform for creating vaccines, for example, against influenza, human immunodeficiency viruses (HIV), Marburg, and Ebola viruses [35–37]. Several factors are responsible for this: lack of nAbs against VSV in humans; a small and easy-to-manipulate genome; cytoplasmic replication without risk of integration into the host genome. Importantly, VSV is able to replicate in a variety of cell lines *in vitro*, yielding high titers and allowing for large-scale virus manufacturing [38–41]. Additional VSV benefits include its ability to replicate in the hypoxic tumor microenvironment (TME) [42,43] and induce tumor cell pyroptosis *via* activation of the caspase-3/gasdermin E (GSDME) axis with an antitumor response [44,45]. Also, VSV has a naive tropism towards cells expressing a small amount of type I interferons (IFNs) or having defective signal transduction, such as cancer cells [36]. Several VSV-based preclinical studies demonstrated promising results in the therapy of various types of cancer (Table 1, Ref. [46–57]).

However, some studies show that the selectivity of unmodified, wild-type VSV (WT-VSV) is often insufficient, which leads to strong neurotoxicity [58]. Therefore, efforts are being made to optimize VSV-based therapies. In this review, we will discuss recent advances in the development of safer and more effective VSV therapies based on the modification of the viral genome and their combination with other traditional treatments.

## 2. VSV Modifications to Improve Oncolytic Properties

WT-VSV needs to be modified in order to improve its oncoselectivity and safety without compromising its oncolytic abilities. Several approaches can be used to create a truly effective VSV-based OV: (i) modification of M, which affects its ability to inhibit antiviral response in normal cells; (ii) introduction of mutations into G that limit or direct VSV tropism; (iii) virus pseudotyping for inhibition of VSV neurotropism; (iv) VSV attenuation *via* disruption of the normal gene order; (v) using conditionally replicating VSV; (vi) using VSV encoding immunostimulatory genes; (vii) introduction of microRNA targets into the VSV genome for inhibition of VSV-induced toxicities [40,59] (Fig. 2). Here, we provide updates on some of these strategies and discuss new developments.

### *2.1 Modification of Viral Proteins*

The oncoselectivity of WT-VSV is not sufficient since M is able to inhibit the effects of type I IFN, which leads to its accumulation in the nuclear envelope, inhibition of mRNA transport into the cytoplasm, and a decrease in the expression of antiviral genes in both healthy and malignant infected cells [60]. For this reason, WT-VSV exhibits strong toxicity, especially neurotoxicity, in mice and primates [61–63]. This problem can be solved by introducing mutations into M. VSV with a mutation or deletion of methionine at position 51 of the M (M51) inhibits the binding of the M to the mRNA complex and is unable to inhibit the antiviral response in infected cells. At the same

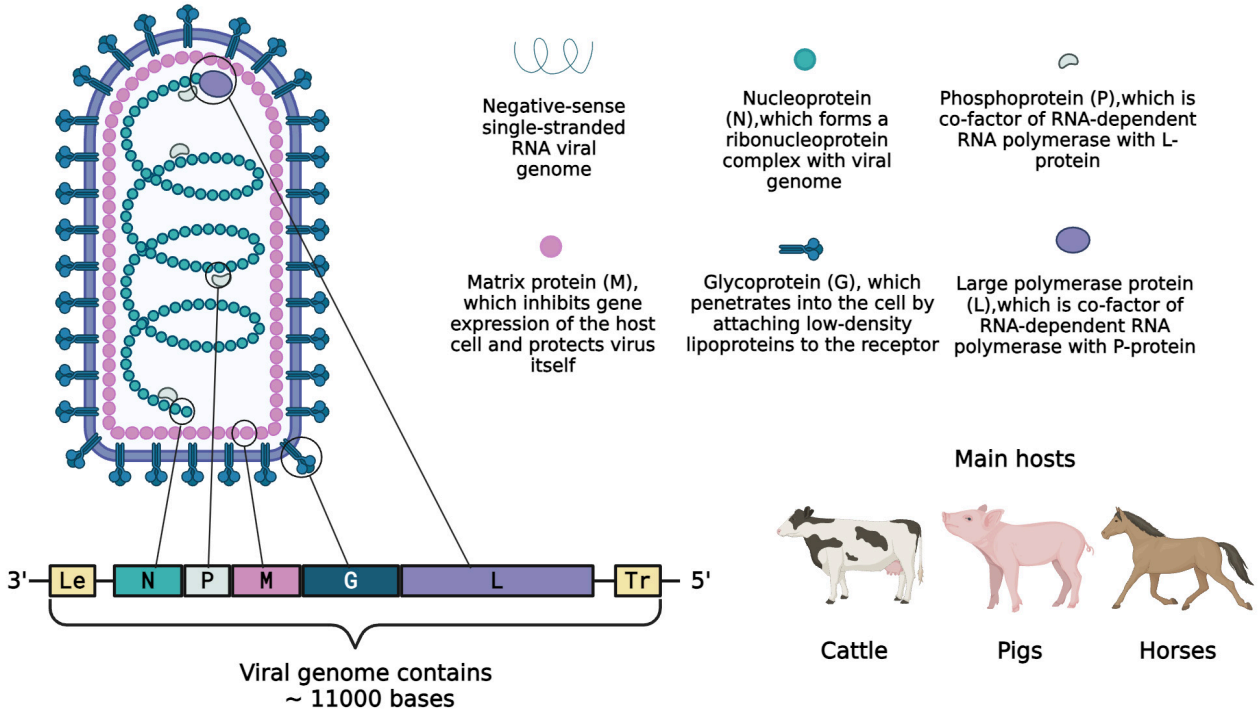


**Fig. 1. A schematic presentation of the vesicular stomatitis virus (VSV) structure.** The VSV genome consists of five genes, each responsible for different functions. The natural hosts of VSV are cattle, pigs, and horses. Le, leader sequence; Tr, terminal trailer sequence. Figure was created with Biorender (https://www.biorender.com/).

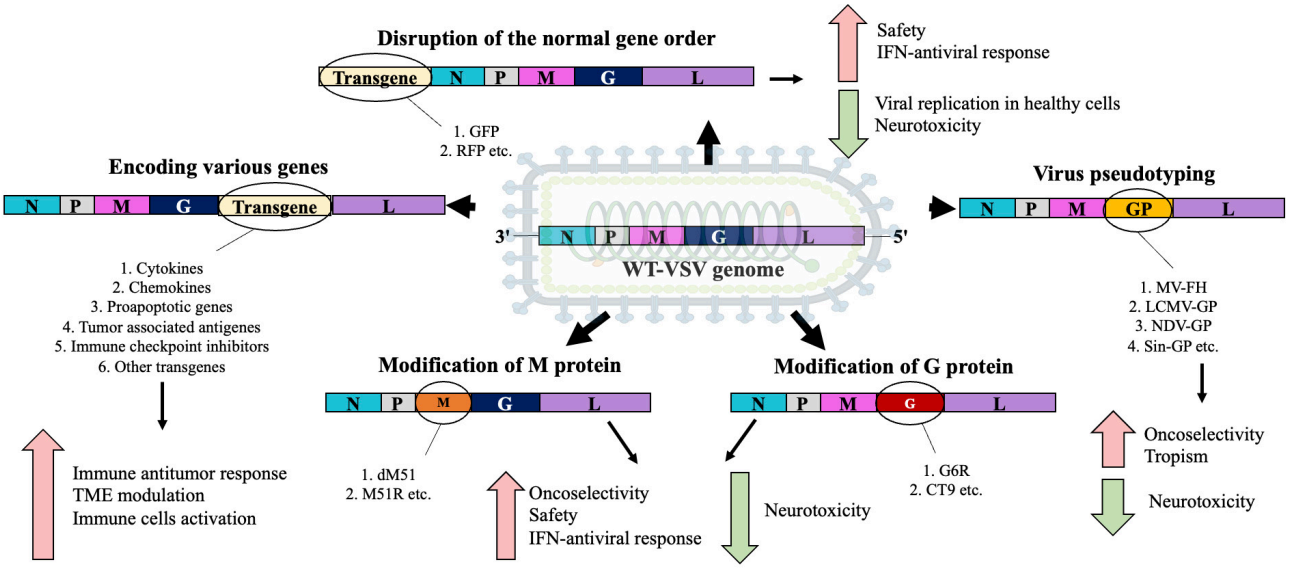


**Fig. 2. The common strategies for VSV modifications.** The VSV genome consists of five genes, each responsible for different functions. The manipulation of the VSV genome can enhance the efficacy of treatment. RFP, red fluorescence protein; GFP, green fluorescence protein; GP, glycoprotein; IFN, interferon; TME, tumor microenvironment; MV-FH, F and H proteins of the measles virus; LCMV-GP, lymphocytic choriomeningitis virus glycoprotein; NDV-GP, Newcastle disease virus glycoprotein; Sin-GP, Sindbis virus glycoprotein; G6R, G protein with replacement of glutamic acid to glycine in the 238 position; CT9, reduction of the G protein's cytoplasmic domain from 29 to 9 amino acids. Figure was created with Biorender (https://www.biorender.com/).

**Table 1. Preclinical effectiveness of VSV against different cancer types.**

| Modified VSV | Type of cancer | Animals | References |
|---|---|---|---|
| VSV-hIFN$\beta$ | Hepatocellular carcinoma | Buffalo rats | [46] |
| VSV-M51R | Pancreatic ductal adenocarcinoma | Naked mice | [47] |
| VSV-$\Delta$M51-GFP | Prostate cancer | Nude mice | [48] |
| VSV-M51R | Colorectal cancer | Balb/c nude mice | [49] |
| VSV-M51R | Neuroendocrine tumor | Athymic nude mice | [50] |
| VSV-$\Delta$M51 | Glioblastoma | CD-1 nude mice | [51] |
| VSV-mIFN$\beta$, VSV-$\Delta$M51-NIS | Endometrial cancer | Athymic mice | [52] |
| VSV-mIFN$\beta$ and VSV-GFP | Non-small cell lung cancer | NCI nu/nu nude mice | [53] |
| VSV-IFN$\beta$ | Multiple myeloma | C57Bl/KaLwRij mice | [54] |
| VSV-IL12 | Squamous Cell Carcinoma | C3H/HeJ mice | [55] |
| VSV-M51R | Breast cancer | BALB/c WT, nude mice | [56] |
| VSV-M51R | Malignant melanoma | Athymic C57BL/6-nu/nu mice | [57] |

$\Delta$M51, M with deletion of methionine in position 51; M51R, M with substitution of methionine in position 51 to arginine; GFP, green fluorescent protein; IL, interleukin; NIS, sodium iodide symporter; hIFN$\beta$, human interferon $\beta$; mIFN$\beta$, mouse interferon $\beta$; WT, wild type; NCI, National Cancer Institute.

time, virus replication in most malignant cells with a disrupted IFN pathway is not limited [64]. VSV, in which methionine in position 51 of M was replaced with arginine, showed its effectiveness *in vivo* on a model of pancreatic adenocarcinoma with intratumoral administration of $1 \times 10^8$ PFU to unstimulated nude mice, which were implanted with Panc 03.27 [47]. The authors suggested that the generation of tumor antigens as a result of VSV-mediated cell lysis could lead to the stimulation of adaptive immunity. Activated natural killer (NK) cells and other antigen-presenting cells (APCs) may then prime T and B lymphocytes against the remaining viable tumor cells. In another study, VSV with the M51 mutation also showed its effectiveness *in vivo* against colorectal cancer with an intra-tumoral injection of $6 \times 10^8$ PFU. Interestingly, malignant cells retained some degree of IFN signalling, which, however, did not negatively impact the VSV-mediated oncolytic effect in sensitive cells [49]. The VSV with the M51 deletion, similar to the M51 mutation, also showed *in vitro* efficacy against 15 human glioblastoma cell lines obtained by surgical resection *ex vivo*. In addition, such therapy showed *in vivo* efficacy without signs of neurotoxicity when administered intratumorally at $1 \times 10^7$ PFU to mice carrying malignant glioma cells U87 or U118 [51].

While modification of the M by methionine substitution or deletion is quite common and mainly found in combination with other modifications of the viral genome [39], the viral oncoselectivity can also be improved *via* the generation of chimeric VSV recombinants encoding either modified VSV G or heterogeneous glycoproteins. The G is responsible for receptor recognition, cell entry, and viral fusion, making it the major target for eliciting a humoral immune response [65]. Heterogeneous glycoproteins are used to improve tropism towards specific types of malignant cells. For example, a study where the G was replaced by the F and H proteins of the measles virus (MV) (VSV-FH) demonstrated complete hepatocellular carcinoma regression (Hep3B model) in 70% of cases with intratumoral administration of $1 \times 10^7$ median tissue culture infectious dose (TCID50) VSV-FH in unstimulated nude mice [66]. In addition, this virus variant demonstrated the absence of hepatotoxicity and neurotoxicity due to the lack of CD46 expression on neuronal cells [67]. This study presented a treatment option for classical Hodgkin's lymphoma characterized by the presence of CD30 expression. VSV-CD30 was produced by replacing the VSV G with the sequence encoding the FHmut-CD30scFv fusion protein. This modified virus demonstrated increased survival and inhibition of tumor growth in the KM-H2 xenograft model in NOD scid gamma (NSG) mice with both intratumoral ($3 \times 10^6$ or $3 \times 10^8$ TCID50) and intravenous ($3 \times 10^8$ TCID50) administration [68].

Another popular approach is to replace the VSV G with the glycoprotein of the lymphocytic choriomeningitis virus (LCMV) (VSV-GP). This virus has proven its safety when administered systemically, intracranially, and intratumorally to immunodeficient mice [69–71] and demonstrated effectiveness in the syngeneic melanoma model B16-OVA [72]. VSV-GP study on various prostate cancer models have also demonstrated significant results. For instance, two intratumoral injections of $1 \times 10^7$ PFU caused total regression of Du145-induced tumors, one intratumoral injection of $2.3 \times 10^8$ PFU had the same effect on 22Rv1-induced tumors, and three intratumoral injections of $1 \times 10^8$ PFU led to a significant increase in median survival and decrease in tumor growth in the syngenic TRAMP-C1 model [73].

An interesting modification of the VSV glycoprotein is its replacement with the Sindbis virus glycoprotein (Sindbis GP), modified to contain the Fc-binding domain of the *Staphylococcus aureus* protein A (Sindbis-ZZ). This virus demonstrated *in vitro* efficacy on human breast

cancer cells in the presence of human monoclonal antibodies directed against the Her2/neu receptor [74]. VSV-gp160G, which contains HIV-1 gp160 in place of the VSV G protein, demonstrated a high efficiency against adult T-cell leukemia, an aggressive cancer of $CD4^+/CD25^+$ T cells caused by T-cell human lymphotropic virus (HTLV-1). VSV-gp160G, which targets only $CD4^+$ cells, showed safety when tested in healthy human $CD4^+$ T cells *ex vivo*, as well as inhibited tumor growth and metastasis in the TLO-m1-luc NSG mouse model when administered intraperitoneally at two doses of $2 \times 10^6$ PFU and one dose of $1 \times 10^7$ PFU of virus with no neurotoxicity observed [75].

The introduction of mutations in the G can also be used to limit VSV tropism. For example, the VSV G6R mutant induces type I IFN responses more efficiently than M51R [76], causing a heightened anti-inflammatory response; however, the antitumor response to the M51 variant was more diverse and strong [59,77].

There is also data available about the optimal VSV phenotype against certain types of cancer and viral attenuation as a result of foreign gene insertion upstream of the viral genes. For example, VSV-CT9-M51 with an M deletion and VSV-p1-GFP expressing GFP in position 1 of the genome, therefore shifting viral genes downstream, were effective in the treatment of glioblastoma [78]. In another study, reduction of the G protein's cytoplasmic domain from 29 to 9 amino acids in VSV-CT9 and expression of the HIV envelope protein were responsible for virus safety in mice [79]. In the case of VSV-p1-GFP, the sequence encoding GFP, inserted before viral genes, reduced virulence [80]. VSV-p1-GFP showed the highest efficiency when administered intravenously at a dose of $5 \times 10^6$ PFU to SCID mice implanted with U87 cells in the striatum of the brain. Moreover, VSV-p1-GFP infection led to not only tumor volume reduction but also small tumor islets, which is a significant clinical achievement [78].

To improve the safety profile, VSV can be attenuated by changing the normal order of viral genes, taking into account the fact that the level of gene expression is position-dependent, with gene located at the $3'$ end of the genome ($1'$ gene position) expressed at the highest level, as was shown in the case with VSV-p1-GFP [80,81]. Another study demonstrated that all Swiss/Webster mice in the group that received intranasal administration of $5 \times 10^4$ PFU of VSV-12′GFP (with normal gene order) survived, while all animals in the group that received VSV-G/GFP died from neurotoxicity [81]. In comparison to the faster-replicating unmodified VSV, insertion of two genes at the $3'$ terminus of the viral genome resulted in a highly attenuated viral phenotype in terms of growth kinetics *in vitro* and improved tolerance *in vivo*. As a result, the slower rate of viral replication protected the nearby uninfected cells by allowing infected cells more time to upregulate an IFN-based antiviral response, which can further slow down the spread of VSV [81].

Normal gene order can also be disrupted without transgene insertion but by rearrangement of sequences, encoding necessary for viral replication proteins. For instance, the median lethal dose (LD50) of VSV, administered intranasally to Swiss-Webster mice, increased 3000-fold after *N* gene transposition to the $4'$ position, while the positions of other genes remained intact as compared to WT-VSV. In addition, a significant decrease in viral replication level and side effects associated with neurotoxicity were observed [82]. However, the location of the sequence encoding G must be considered as heightened virus-neutralizing antibody production was demonstrated as a result of *G* gene transposition to the $1'$ position and *N* gene transposition to the $4'$ position [83]. Production of antibodies to G, containing major viral epitopes, can lead to resistance and rapid clearance of VSV-based therapy, significantly decreasing its efficacy. To summarise, although there are only several studies on VSV attenuation *via* disruption of the normal gene order, this approach has great prospects for the treatment of malignancies of the central nervous system and brain, which are limited by viral neurotoxicity.

### *2.2 VSV Genome Modification: Insertion of Additional Exogenous Genes*

The most popular gene insertion in the VSV genome is IFN-$\beta$. IFN-$\beta$ stimulates the innate immune response in healthy cells, but not in cancer cells, since they have a defect in the production of type I IFN, while also stimulating the antitumor response [46,53]. It has been proven that VSV expressing IFN-$\beta$ is capable of selective replication in tumor cells and induction of their death precisely due to defective innate immune responses [84]. A lot of studies have shown that VSV-IFN-$\beta$ shows no signs of neurotoxicity [46]. While different types of INFs were demonstrated to be capable of protecting healthy cells from exposure to the virus, IFN-$\alpha$-2a showed much greater efficacy in cancer cells than IFN-$\beta$ but had the same effect on healthy cells [85]. For instance, intratumoral injection of $1 \times 10^7$ TCID50 or intravenous injection of $1 \times 10^6$ TCID50 VSV-hIFN-$\beta$ were well-tolerated by athymic mice [52]. However, due to the fact that hIFN-$\beta$ was not biologically active in mice, many mice died of neurotoxicity after intratumoral but not intravenous administration, which indicates the spread of the virus following intratumoral administration. Despite the observed toxicity, complete tumor regression was observed in both cases. Importantly, VSV-mIFN-$\beta$ showed high efficacy *in vivo*, with a 3-fold increase in median survival and protection against readministration of cancer cells in a model of non-small cell lung cancer [53]. In addition, VSV-hIFN-$\beta$ showed its effectiveness and complete safety with a single intratumoral and intravenous administration of $5 \times 10^8$ PFU on day 21 post-transplantation of squamous cell carcinoma FAT7 cells to immunocompetent Fischer-344 rats to create a model of squamous cell carcinoma of the head and neck [86].

Another alternative strategy for VSV modification is the addition of the sodium iodine symporter (NIS), which allows the use of special radio tracers for non-invasive imaging of the virus bio-distribution [87]. Despite the fact that VSV-IFN-$\beta$ and VSV-IFN-$\beta$-NIS have a higher safety profile than WT-VSV, even these virus variants were demonstrated to cause extremely strong neurotoxicity once myeloma metastasized to the central nervous system [88].

In addition to IFN-$\beta$, there are other proteins that enhance viral anti-tumor qualities. Host antitumor immune responses can be enhanced by VSV-mediated delivery of proinflammatory cytokines, molecules stimulating the activity of T cells, tumor-associated antigens (TAA), immune checkpoint inhibitors (ICI), and cell suicide genes [38,89,90]. The main immune-stimulating transgenes and their delivery by OVs, including VSV, are shown in Table 2 (Ref. [55,68,91–180]).

Incorporation of pro-inflammatory cytokines in the viral genome is a promising approach since many of them play an important role in attracting immune cells to the tumor [181]. Frequently used immunostimulating genes include *GM-CSF*, *TNF-*$\alpha$, various *ILs* (*IL-2*, *IL-12*, *IL-15*, *IL-18*, *IL-21*, and *IL-24*), and chemokines (such as *CCL5*, *CCL20*, *CCL21*, *CXCL4L1*, and *CXCL10*) [38,130,182].

One of the successful applications of modified VSV delivering murine IL-12 was the daily intravenous administration of $1 \times 10^7$ PFU of VSV-mIL12 for 5 days to immunocompetent C3H/HeJ mice in the squamous cell carcinoma (SCC) VII neck and head carcinoma model [55]. A significant decrease in the tumor and an increase in the median survival rate were demonstrated in comparison with the VSV-F encoding fusion protein in the absence of toxicity. The use of VSV encoding chemokines yielded some controversial results that require further research. For example, intravenous administration of $5 \times 10^7$ TCID50 of VSV-mCXCL9 resulted in an increase in survival and an inhibition of tumor growth in a syngeneic plasmocytoma model 5TGM1, but did not show a significant difference compared to PBS in a syngeneic model of non-small cell lung cancer LM2 with an intramural administration of $5 \times 10^8$ TCID50 [132]. VSV encoding human IL-15 (VSV-$\Delta$M51-opt.hIL-15) demonstrated its effectiveness in primary and metastatic colon tumor CT26 in a Balb/c mouse model with intravenous administration of three doses of $5 \times 10^8$ PFU of the virus. It is worth mentioning that VSV-GFP was powerless against tumor metastases in the lungs, which indicates the effect of IL-15 in attracting immune cells [114]. The murine gammaherpesvirus 68 *M3* gene encodes a protein that binds to a wide range of chemokines (C, CC, CCC, and CX3C) with high affinity [183]. Intrahepatic administration of rVSV(M$\Delta$51)-M3, expressing the secreted M3 form, at doses of $5 \times 10^8$ and $5 \times 10^9$ PFU, led to long-term remission in 40% and 50% of Buffalo rats with hepatocellular carcinoma McA-RH7777, respectively [64].

In line with the described strategies, some VSVs were modified to express tumor-suppressor or proapoptotic genes, such as *p53*, *PTEN*, *p16*, *Rb*, *TRAIL*, and *Smac* [38,140,142]. As many types of malignancies have a p*53* *m*utation, expression of these transgenes in cancer cells can enhance OV efficacy and promote tumor apoptosis. VSV-M(mut)-mp53, where amino acids DTY in the 52–54 position of the M were substituted with alanins, showed efficacy in a model of breast adenocarcinoma TS/A in BALB/c mice with intravenous administration of the virus at a dose of $5 \times 10^7$ PFU [136]. Less than 50% of mice died within 120 days; however, increasing the dose to $5 \times 10^8$ PFU decreased the median survival rate to 75 days. Smac mimetics have been proven to increase the sensitivity of tumor cells to antitumor therapy. VSV encoding the *Smac* gene (VSV-S) showed its enhanced activity in the 4T1 BALB/c mouse breast cancer model with a three-fold intratumoral injection of $3 \times 10^6$ PFU and in the T-47D breast cancer model with a single injection of $1 \times 10^5$ PFU [144]. It was demonstrated that VSV therapy led to a decrease in tumor volumes and the degradation of malignant tissues. And last but not least, anti-angiogenesis, one of the popular strategies for antitumor effects, relies on the expression of a vascular endothelial growth factor inhibitor (VEGFI) and has already shown promising results [145].

In addition to the discussed approaches, OVs armed with relevant TAA also provide promising therapeutic strategies for the treatment of solid tumors by inducing a potent and persisting systemic antitumor response. Claudin-6, carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), and human dopachrome tautomerase (hDCT) are some of the widely used targets [140,147,149,184]. CD133 is the most studied marker of various carcinomas [185]. VSV-CD133 therapy resulted in increased survival of NOD/SCID mice inoculated with HuH7 hepatocellular carcinoma cells [151]. Only mice that were intratumorally injected with 4 doses of $1 \times 10^6$ TCID50 of VSV-CD133 remained alive after 40 days of the study. However, all mice treated with VSV-CD133 intracranially during implantation of NCH644 glioma spheres had to be killed on day 8 due to severe neurotoxicity, which sets certain restrictions for the use of VSV.

ICI, such as PD-L1, CTLA-4, and CD40, can be effectively used to allow T cells to exert their cytotoxic activity, which can usually be inhibited due to the aberrant function of malignant cells [38]. There are also several successful studies on the costimulating members of the TNF receptor superfamily, such as OX40L, CD30, CD40L, and 4-1BB [159,186]. Interestingly, VSV delivering CD40L did not show an increased antitumor response compared to VSV-GFP in the B16OVA murine melanoma model, although the use of AdV encoding CD40L helped cure 75% of C57BL/6 mice. Each group of animals received six intratumoral injections of either virus therapy: (i) replication-active VSV-GFP ($5 \times 10^8$ PFU), (ii) VSV-CD40L ($5 \times 10^8$

**Table 2. Examples of recombinant OV-mediated delivery of transgenes.**

| Transgene | Examples | Reference |
|---|---|---|
| Immunostimulatory factors | | |
| GM-CSF | VV-GM-CSF | [91,92] |
| | HSV-GM-CSF (T-Vec) | [93] |
| | AdV-GM-CSF | [94] |
| | VSV-GM-CSF | [95] |
| TNF-$\alpha$ | AdV-TNF-$\alpha$ | [96,97] |
| | VSV-TNF-$\alpha$ | [98] |
| IL-2 | AdV-IL-2 | [96,99] |
| | HSV-IL-2 | [100] |
| | VV-IL-2 | [101] |
| IL-12 | AdV-IL-12 | [102,103] |
| | MeVac-IL-12 | [104] |
| | MG1-IL-12 | [105] |
| | NDV-IL-12 | [106] |
| | SFV-IL-12 | [107] |
| | Sin-IL-12 | [108] |
| | VV-IL-12 | [109] |
| | VSV-IL-12 | [55] |
| IL-15 | VV-IL-15 | [110,111] |
| | AdV-IL-15 | [112] |
| | DelNS1-IL-15 | [113] |
| | VSV-IL-15 | [114] |
| IL-18 | HSV-IL-18 | [115] |
| IL-21 | VV-IL-21 | [116] |
| IL-23 | VV-IL-23 | [117] |
| | VSV-IL-23 | [118] |
| IL-24 | AdV-IL-24 | [119] |
| | VV-IL-24 | [120,121] |
| | NDV-IL-24 | [122] |
| IFN-$\beta$ | VV-IFN-$\beta$ | [123] |
| | AdV-IFN-$\beta$ | [124] |
| | VSV-IFN-$\beta$ | [125] |
| Chemokines | | |
| CCL5 | AdV-CCL5 | [126] |
| CCL20 | AdV-CCL20 | [127,128] |
| CCL21 | AdV-CCL21 | [129] |
| CXCL4L1 | H-1 PV-CXCL4L1 | [130] |
| CXCL10 | AdV-CXCL10 | [131] |
| | H-1 PV-CXCL10 | [130] |
| CXCL9 | VSV-CXCL9 | [132] |
| | AAV-CXCL9 | [133] |
| Proapoptotic genes | | |
| p53 | AdV-p53 | [134] |
| | NDV-p53 | [135] |
| | VSV-p53 | [136] |
| PTEN (P10) | HSV-P10 | [137] |
| | AdV-PTEN | [138] |
| p16 | SVA-p16 | [139] |
| TRAIL | Ad-TRAIL | [140,141] |
| | NDV-TRAIL | [142] |
| Smac | VV-Smac | [143] |
| | VSV-Smac | [144] |

**Table 2. Continued.**

| Transgene | Examples | Reference |
|---|---|---|
| Antiangiogenesis genes | | |
| VEGI | AdV-VEGI | [145] |
| VEGF | AdV-VEGF | [146] |
| Tumor associated antigens | | |
| Claudin-6 | MV-CLDN6 | [147] |
| CEA | AdV-CEA | [140] |
| | MV-CEA | [148] |
| PSA | AdV-PSA | [149] |
| hDCT | AdV-hDCT | [150] |
| | VSV-hDCT | |
| CD133 | MV-CD133 | [151] |
| | AdV-CD133 | [152] |
| | VSV-CD133 | [151] |
| Immune checkpoint inhibitors | | |
| PD-L1 | VV-iPD-L1 | [153] |
| | MV-a-PD-L1 | [154] |
| | HSV-a-PD-L1 | [155] |
| | AdV-a-PD-L1 | [156] |
| | VSV-a-PD-L1 | [157] |
| Immune co-stimulatory genes | | |
| OX40L | NDV-OX40L | [158] |
| | AdV-OX40L | [159,160] |
| | IAV-OX40L | [161] |
| CD30 | MV-CD30 | [68] |
| | VSV-CD30 | |
| CD40L | AdV-CD40L | [160,162,163] |
| | HSV-CD40L | [164] |
| | VV-CD40L | [165] |
| | VSV-CD40L | [166] |
| 4-1BBL | VV-4-1BBL | [167,168] |
| Cell suicide genes | | |
| HSV-TK | VV-HSV-TK | [169] |
| | AdV-HSV-TK | [170,171] |
| | VSV-HSV1-sr39tk | [172] |
| Cytosine deaminase (CD) | HSV-1-yCD | [173] |
| | VG9-CD | [174] |
| | MV-CD | [175] |
| | VSVΔ51-CD::UPRT | [176] |
| Nitroreductase (NTR) | HSV-NTR (HSV1790) | [177] |
| | AdV-NTR | [178,179] |
| Cytochrome P450 | HSV-P450 | [180] |

VV, Vaccinia virus; GM-CSF, granulocyte-macrophage colony-stimulating factor; HSV, Herpes simplex virus; AdV, Adenovirus; VSV, Vesicular stomatitis virus; TNF, tumor necrosis factor; IL, interleukin; MeVac, Measles Schwarz vaccine; MG1, Maraba MG1 virus; NDV, Newcastle disease virus; SFV, Semliki Forest virus; Sin, Sindbis virus; DelNS1, Influenza A virus with deleted NS1 gene; IFN, Interferon; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; H-1 PV, H-1 parvovirus; AAV, Adeno-associated virus; PTEN, phosphatase and tensin homolog; SVA, Senecavirus A; TRAIL, TNF-related apoptosis-inducing ligand; Smac, second mitochondria-derived activator of caspases; VEGI, vascular endothelial growth inhibitor; VEGF, vascular endothelial growth factor; MV, Measles virus; CEA, carcinoembryonic antigen; PSA, prostate-specific antigen; hDCT, human dopachrome tautomerase; CD, cluster of differentiation; PD-L1, programmed death-ligand 1; OX40L, tumor necrosis factor receptor superfamily, member 4 (TNFRSF4) ligand; IAV, Influenza A virus; 4-1BBL (4-1BB ligand), Tumor necrosis factor ligand superfamily member 9; HSV-TK, Herpes simplex virus thymidine kinase; HSV1-sr39tk, mutant herpes simplex virus thymidine kinase; VG9, Vaccinia virus strain Guang9; UPRT, uracil phosphoribosyltransferase.

PFU), (iii) replication-defective AdV-GFP ($1 \times 10^9$ PFU), or (iv) AdV-CD40L ($1 \times 10^9$ PFU). CD40L expression was expected to lead to an increase in $CD4^+$ and $CD8^+$ tumor-specific T cell numbers. While the doses used for both viruses were similar, the difference in the outcome may be due to the speed with which these OVs induce a pro-inflammatory response. VSV induces a rapid inflammatory response in malignant cells, activation of tumor-nonspecific $CD8^+$ T cells in the spleen, and strong expression of pro-inflammatory cytokines, especially IFN-$\gamma$, for several days post-treatment. It was demonstrated that mainly viral epitopes are presented to naïve T cells as antigens; therefore, the anti-tumor effect after VSV-CD40L administration is indistinguishable from VSV-GFP. The effect of AdV can only be observed several days post-administration, and AdV-expressed CD40L is able to carry out its costimulatory role for TAA-specific T cells and lead to increased tumor inhibition compared to AdV-GFP. This shows the importance of the right choice of OV for successful therapy depending on the type of cancer [166].

Suicide genes are also interesting because they can be safely expressed in a cell, after which they cause cell death by apoptosis upon activation by a drug or antibody, which can create an effective combination therapy. In addition, these genes are able to infect neighbouring tumor cells, preventing metastasis [38]. These genes include, among others, *HSV-TK*, cytosine deaminase, the fusion *FCU1* gene (containing CD and uracil-phosphoribosyltransferases), nitroreductase, and cytochrome P450 [187–189]. VSV-HSV1-sr39tk, encoding an improved version of the thymidine kinase reporter (sr39tk) of HSV-1, demonstrated the possibility of highly sensitive *in vivo* imaging using positron emission tomography (PET) on a model of hepatocellular carcinoma in Buffalo rats [172]. However, in the future, this virus may be used in combination therapy with the prodrug ganciclovir (GCV), which will enhance the antitumor response.

An important and effective strategy using the suicide gene is the expression of the fusion enzyme cytosine deaminase/uracylphosphoribosyltransferase (CD/UPRT) and the systemic delivery of non-toxic 5-fluorocytosine (5FC), which is deaminated by enzymes and converted into the chemotherapeutic drug 5-fluorouracil (5FU) [176]. In this case, the effectiveness of VSV-C (VSV$\Delta$51-CD::UPRT) in combination with 5FC was demonstrated in models of breast adenocarcinoma TSA or T-cell lymphoma EG7 with a double intravenous injection of $2 \times 10^7$ PFU with optimization of 5FC administration to achieve a synergistic effect without signs of toxicity. This combination therapy approach led to more significant tumor growth inhibition and increased animal survival than both drugs administered separately [190].

Another promising option relies on small transmembrane proteins associated with reovirus fusion (FAST). For example, VSV, encoding the FAST p14 protein (VSV-p14), demonstrated enhanced antitumor activity in comparison with VSV-GFP in models of primary and metastatic breast cancer and metastatic colon cancer. The study was conducted on BALB/c mice, which were transplanted with cells of triple-negative breast cancer 4T1 and metastatic colon carcinoma CT26, followed by intravenous administration of three doses of $1 \times 10^8$ PFU and $1 \times 10^7$ PFU of VSV-p14 or VSV-GFP, respectively. An increase in survival and a decrease in the number and size of metastases were observed as a result of this successful therapy [191].

An interesting decision is miRNA delivery by VSV, which has a negative-sense single-stranded RNA genome. VSV, encoding miRNA-143, which is involved in the process of distant osteosarcoma metastasis [192], demonstrated a strong cytopathic effect on the mouse (LM8) and human osteosarcoma (143B, HOS, Saos-2, and MG-63) cells *in vitro*. Regretfully, the same effect was not achieved in an *in vivo* experiment [193,194].

The use of VSV modifications leading to activation of the host immune system is a promising approach that can drastically change the effectiveness of OV therapy. The majority of studies are shown in Table 3 (Ref. [53–55,64,86,95,114,118,132,136,144,151,166,190,191,195,196]).

### *2.3 VSV Modifications by Directed Evolution*

Recombinant VSVs with enhanced oncoselectivity can be generated *via* directed evolution, which adapts viruses to malignant cells. In a recent study, VSV-$\Delta$M51 demonstrated significantly increased fitness and cytotoxicity against mouse embryonic fibroblasts (MEF) with *p53* knockout and low adaptation to MEF cells without *p53* knockout after 40 passages. Analysis of the semi-maximal effective dose (EC50) showed that the viruses obtained by evolution were more effective against the p53-deficient breast cancer model (4T1) but had no advantage compared to WT-VSV against the p53-positive colon cancer model (CT26) [197]. One of the evolved viruses was particularly effective against p53-deficient 4T1 cells with two injections of $1 \times 10^8$ PFU of the virus into BALB/c mice. This L3 virus contained a G1456U mutation in the *P* gene and a G5129A mutation in the *L* gene, which shows the evolutionary nature of its effectiveness in the p53-/- cancer cell line [197].

Also, directed evolution of VSV-G-GFP in human glioblastoma cells after positive selection during multiple passages produced the VSV-rp30a mutant with four mutations compared to VSV-G-GFP [198]. The intravenous administration of $5 \times 10^7$ PFU of the VSV-rp30a variant to SCID mice in a xenograft model of Ewing's sarcoma (A673 cells) led to enhanced targeting of a multifocal glioblastoma tumor in a mouse xenograft model as well as an 11-fold decrease in tumor volume compared to VSV-G-GFP [199,200].

**Table 3. Examples of VSV modified by transgene insertion.**

| Virus | Type of cancer | Dose | Animals | Results | Reference |
|---|---|---|---|---|---|
| VSV-mIFN$\beta$ | Non-small cell lung cancer (LM2 cell line) | Three IT doses of 6.6 × $10^8$ TCID50 | A/J mice | Tumor regression, 3-fold increase in median survival, cure of 30% of mice and protection against repeated inoculation of cancer cells, increased numbers of $CD8^+$ and infiltrating regulatory T (Treg) lymphocytes, PD-L1 expression in tumors. | [53] |
| VSV-mIFN$\beta$, VSV-hIFN$\beta$ | Multiple myeloma (5TGM1 cell line) | IV dose of 1 × $10^8$ TCID50 | C57Bl/KaLwRij mice | 100% tumor regression for VSV-mIFN$\beta$ and 80% tumor regression for VSV-hIFN$\beta$. | [54] |
| VSV-hIFN$\beta$ | Squamous cell carcinoma (FAT7 cell line) | IT/IV dose of 5 × $10^8$ PFU | Fischer-344 rats | Tumor regression, complete safety, higher levels of VSV-IFN-$\beta$ RNA in tumors compared to non-malignant tissues. | [86] |
| VSV-mIL12 | Neck and head carcinoma (SCC VII cell line) | IV dose of 1 × $10^7$ PFU for 5 days | C3H/HeJ mice | Significant decrease in tumor size, 40% of VSV-IL12-treated mice are alive and tumor-free for 100 days. | [55] |
| ΔM51 VSV/opt.hIL-15 | Primary and metastatic colon tumor (CT26 cell line) | Three IV doses of 5 × $10^8$ PFU | Balb/c mice | Effective on primary colon tumor, but powerless against tumor metastases, enhanced anti-tumor $CD8^+$ T-cell response and increase in NK cell numbers. | [114] |
| VSVdM51-mGM-CSF | Bladder cancer (MB49 cell line) | IV dose of 5 × $10^8$ PFU | C57Bl/6 mice | Tumor regression, enhanced release of immunogenic DAMPs, polarization of monocytes toward an M1-like phenotype, and increased NK- and $CD8^+$ T-cell migration. | [95] |
| VSV23 (VSV-IL-23) | Breast cancer (JC cell line) | IT dose of 1 × $10^7$ PFU | BALB/c mice | Reduction in tumor size, infiltration of $CD8^+$ and $CD4^+$ T cells, macrophages, and neutrophils. | [118] |
| VSV-mCXCL9 | Syngeneic plasmacytoma (5TGM1 cell line) | IV dose of 5 × $10^7$ TCID50 | C57BL/KaLwRij mice | Increase in survival and decrease in 5TGM tumor growth, but no significant difference compared to control in LM2 tumor, 10-fold increase in CXCL9 concentration in tumors compared to blood. | [132] |
| | Non-small cell lung cancer (LM2 cell line) | IT dose of 5 × $10^8$ TCID50 | A/J mice | | |
| VSV(MΔ51)-M3 | Hepatocellular carcinoma (McA-RH7777 cell line) | IH dose of 5 × $10^8$ or IH dose 5 × $10^9$ PFU | Buffalo rats | Significant reduction in accumulation of neutrophils and NK cells in tumors, a 2-log elevation of intratumoral viral titer, significant tumor regression and long-term remission of 40% (5 × $10^8$ PFU) and 50% (5 × $10^9$ PFU) | [64] |
| VSV-M(mut)-mp53 | Breast adenocarcinoma (TS/A cell line) | IV dose of 5 × $10^7$ PFU | BALB/c mice | Tumor regression, high expression of p53 in tumors, enhanced $CD49b^+$ NK and tumor-specific $CD8^+$ T cell responses, less than 50% of all mice died within 120 days. | [136] |
| VSV-S (Smac) | Breast cancer (4T1 cell line) | Three IT doses of 3 × $10^6$ PFU | BALB/c mice | Nearly 60% decrease in tumor progression in the 4T1 tumor model and more than 85% tumor volume reduction, increased apoptosis due to high expression of poly(ADP-ribose) polymerase (PARP), caspase-9, and caspase-3. | [144] |
| | Breast cancer (T-47D cell line) | IT dose of 1 × $10^5$ PFU | Nude mice | | |
| VSV-RGD3, VSV-Echi9 | Plasmacytoma (MPC-11 cell line) | IV dose of 5 × $10^6$ PFU | BALB/c mice | Therapeutic effect, high level of VSV-Echi9 entry into cells when the LDL receptor is effectively masked. | [195] |
| VSV-CD133 | Hepatocellular carcinoma (HuH7 cell line) | 4 IT doses of 1 × $10^6$ TCID50 | NOD/SCID mice | Only VSV-CD133-treated mice with HuH7 tumors remained alive after 40 days, while mice with glioma spheres died on day 8 due to severe neurotoxicity. | [151] |

**Table 3. Continued.**

| Virus | Type of cancer | Dose | Animals | Results | Reference |
|---|---|---|---|---|---|
| | Glioma (NCH644 cell line) | IC dose of 2 × $10^5$ TCID50 | | | |
| rrVSV-G (rrVSV-G-SCA-erbb2) | Breast cancer (D2F2/E2 cell line) | IT dose of 1 × $10^7$ PFU | BALB/c mice | 70% of mice with average-size tumors and 33% of mice with large tumors were cured, protection of mice from repeated tumor cell injection. | [196] |
| VSV-CD40L | Melanoma (B16ova cell line) | IT dose of 5 × $10^8$ PFU | C57BL/6 mice | No difference in antitumor response compared to VSV-GFP. | [166] |
| VSVΔ51-CD::UPRT | Breast adenocarcinoma (TSA cell line) T-cell lymphoma (EG7 cell line) | Two IV doses of 2 × $10^7$ PFU (with 500 mg/kg 5FC 4 times per day for 4 days) | BALB/c mice | Tumor regression without any signs of toxicity. | [190] |
| VSV-p14 | Triple negative breast cancer (4T1 cell line) | IV dose of 1 × $10^8$ PFU | BALB/c mice | Tumor regression, increase in survival and decrease in the number and size of metastases, increase in numbers of activated splenic $CD4^+$, $CD8^+$, and NK cells. | [191] |
| | Metastatic colon carcinoma (CT26 cell line) | IV dose of 1 × $10^7$ PFU | | | |

IV, intravenous; IT, inratumoral; IC, intracranial; IH, *via* the hepatic artery; TCID50, 50% tissue culture infectious dose; DAMPs, damage-associated molecular pattern molecules; NK, natural killer; LDL, low-density lipoprotein; dM51, M with deletion of amino acid in position 51 of matrix protein.

### *2.4 Modifications for Creating Conditionally Replicating Oncolytic Viruses*

In severely ill cancer patients, the potential for uncontrolled OV spread may compromise the patient's safety [36]. One of the ways to increase oncoselectivity and safety is to create conditionally replicating OVs, a process that relies on two fundamentally different approaches [38]. The first approach is based on the removal of viral genes necessary for virus replication, for example, genes in the *E1A* or *E1B* regions for adenovirus-based vectors and HSV-Tymidine Kinase (or g34.5/joint region) for HSV. For example, the E1A promoter can be replaced by a cancer-specific promoter known to be expressed in the targeted indication [90]. In addition, tumor-specific promoters, such as human telomerase reverse transcriptase (hTERT), which is inactive in most healthy cells but is activated in tumor cells, can serve as selective controls of viral transcription [201]. An interesting variant of this approach is the separation of the genes necessary for VSV replication into two plasmid constructs. The combination of VSV*ΔG and VSVΔL-DsRed demonstrated its exceptional efficacy and safety in a human glioblastoma model when two doses of 2.8 × $10^5$ TCID50 of srVSV(ΔG/ΔL) were administered intratumorally to NOD/SCID mice with G62 tumors. Administration of similar doses of WT-VSV led to death from neurotoxicity in 90% of cases, while therapy with srVSV (ΔG/ΔL) resulted in regression of 80% of tumors without any side effects [202].

The second approach is simpler and cheaper and is based on the chemical modification of the viral capsid by a pH-sensitive polymer. This strategy is based on the fact that the pH in tumors is lower than the pH in healthy tissue [203]. However, this approach has its drawbacks: firstly, chemical modifications do not persist after replication; and secondly, the oncolytic activity exerted by OVs can be inhibited due to excessive protection of the genome by the polymer [38]. Covalent modification of VSV-mIFN-$\beta$ with PEG 5000 resulted in an increase in virus persistence in the blood even in the presence of neutralizing antibodies, delivery of the virus to the tumor site, and a decrease in hepatotoxicity. The study was performed on BALB/c mice inoculated with murine plasma cell myeloma cells (MPC 11), followed by intravenous administration of pegylated virus at a dose of 2.5 × $10^8$ TCID50 [204]. Another study demonstrated that exposure of VSV to UV irradiation led to the generation of non-replicating VSV particles [36]. Despite losing its ability to replicate, VSV maintained potent cytotoxicity *in vitro*. Although the exact mechanism of oncoselectivity of such non-replicating VSV particles is unclear, another study showed that ultraviolet (UV)-irradiated VSV-dM51-GFP enhanced tumor growth in an immunocompetent mouse model of pancreatic ductal adenocarcinoma (PDAC) [205].

**Table 4. Examples of VSV in clinical trials.**

| VSV | NTC number | Tumor selectivity | Route of administration | Phase | Status | Sponsor |
|---|---|---|---|---|---|---|
| VSV-IFNbetaTYRP1 | NCT03865212 | Melanoma stage III-IV | IT, IV | I | Active | Mayo Clinic |
| VSV-IFN-beta | NCT01628640 | Refractory Liver Cancer or Advanced Solid Tumors | IT | I | Active | Mayo Clinic, National Cancer Institute |
| VSV-hIFNbeta-NIS | NCT03120624 | Metastatic or Recurrent Endometrial Cancer | IV | I | Active | Mayo Clinic, National Cancer Institute |
| VSV-hIFNbeta- NIS | NCT03017820 | Relapsed or Refractory Multiple Myeloma, Acute Myeloid Leukemia or Lymphoma | IV | I | Recruiting | Mayo Clinic, National Cancer Institute |
| VSV-IFNbeta-NIS | NCT02923466 | Malignant Solid Tumor | IT | I | Completed | Vyriad, Inc |
| VSV-GP128 | NCT04046445 | Stage IV colorectal cancer | IV | I | Active | Amal Therapeutics Boehringer Ingelheim |
| VSV-IFN$\beta$-NIS | NCT03647163 | Solid tumors | IV | I/II | Recruiting | Vyriad, Inc., Mayo Clinic |
| VSV (Revottack) | NCT05644509 | Advanced malignant solid tumors | IV | I | Not yet recruiting | The Affiliated Hospital of Xuzhou Medical University |
| VSV-IFN$\beta$-NIS | NCT04291105 | Colorectal, head and neck carcinoma, and melanoma | IT | II | Recruiting | Vyriad, Inc. |
| VSV-GP154 | NCT05846516 | Pancreatic cancer | IV (injection) | I | Recruiting | Amal Therapeutics, Boehringer Ingelheim |
| VSV-GP (BI 1831169) | NCT05155332 | Advanced or metastatic solid tumors | IT, IV | I | Recruiting | Boehringer Ingelheim |
| VSV-GP (BI 1821736) | NCT05839600 | Advanced solid tumors | IV | I | Recruiting | Boehringer Ingelheim |

## 3. Combination Therapy

Ideally, a new therapeutic approach is expected to improve the outcome; therefore, combination therapy relying on two strategies should enhance the efficiency of these strategies used separately. Based on this premise, the next part of this review will explore how VSV can improve the outcome of standard treatments such as radio-, chemo-, and immunotherapy in both preclinical and clinical studies.

### *3.1 Combination with Radiotherapy*

Radiotherapy (RT) can play an important role in cancer treatment, not only as a palliative therapy but also as part of a preservation strategy. Despite the fact that radiation regimens have improved over time, the locoregional radiotherapy effect is limited in advanced and metastatic stages of cancer [206]. Many studies have demonstrated that the combination of OVs with RT enhances the antitumor effect [207]. While OVs may increase the radiosensitivity of tumor cells, radiation, in turn, may accelerate viral uptake and replication, leading to cell death [208].

For example, the combination of VSV M and RT led to a favorable antitumor immune response [209]. The authors of this study employed an interesting approach: forming a complex of the plasmid encoding VSV M with the DOTAP-cholesterol liposome (Lip-MP). Mice with lung carcinoma or sarcoma received intratumoral or intravenous injections of Lip-MP and radiation, leading to a significant tumor reduction compared to either treatment alone. The authors highlighted five reasons for such an outcome: (i) M and radiation induce tumor cell apoptosis; (ii) radiation improves transfection and transduction efficiency; (iii) injection of the liposome-DNA complex induces nonspecific antitumor activity; (iv) M alone activates the NK response; and (v) Lip-MP increases radiation-induced apoptosis. Another preclinical study provided further evidence that the combination of VSV and RT (followed by a second round of RT 3 days later) could exert a synergistic antitumor effect *via* robust activation of immune cell infiltration [210]. As a result, significant tumor growth inhibition and increased survival in mice with head and neck cancer were observed in the combination treatment group compared to groups that received either treatment alone.

VSV-IFN-$\beta$ in combination with RT was shown to elicit a more potent anticancer effect than either treatment administered alone [211]. The synergism of the combination has been approved in a preclinical study in the subcutaneous PC3 and orthotopic LNCaP prostate xenograft models and a syngeneic RM9 prostate tumor model. During this study, treated RM9 tumor-bearing mice demonstrated an increase in $CD4^+$ and $CD8^+$ T lymphocyte numbers with 100% resistance to repeated tumor challenge. According to the authors, RT enhanced the activity of VSV-mediated oncolysis *via* attenuation of the innate antiviral response, re-

sulting in increased VSV replication and the generation of an adaptive immune response, as evidenced by an increase in $CD8^+$ lymphocyte numbers and antitumor activity. Local tumor irradiation combined with VSV-IFN-$\beta$ injection resulted in tumor cell death *via* direct and systemic activity in conjunction with pronounced antitumor immunity.

### *3.2 Combination with Chemotherapy*

Chemotherapy remains the leader of first-line conventional cancer therapies [5]. Unlike surgery or RT, chemotherapy has a systemic therapeutic effect and remains integral to the treatment of cancer patients with disseminated disease. Recent studies revealed that chemotherapeutics are also capable of inducing immunogenic cell death (ICD) in malignant cells [212,213]. As delivery of OVs to metastatic sites remains challenging, a combination of chemotherapy exerting a systemic effect with local OV therapy is a promising approach that is actively investigated.

In 2013, Hastie *et al*. [214] combined gemcitabine, the standard drug for the treatment of pancreatic cancer, and VSV as a novel approach to cancer therapy. Results revealed that the combination of VSV-GFP with gemcitabine improved antitumor efficacy in an immunocompetent mouse model of pancreatic ductal adenocarcinoma. Based on the preclinical results, another study demonstrated efficacy due to the combination of VSV with LCL161, a Smac mimetic compound and inhibitor of apoptosis antagonist [215]. This approach inhibited tumor growth in 76–9 rhabdomyosarcoma syngeneic mice when VSV or LCL161 were not effective on their own as compared to negative control mice. The effectiveness of this approach relies on OV-mediated induction of cytokine and chemokine secretion leading to cancer cell death, either directly or indirectly, as a result of recruitment and activation of innate and adaptive immune cells targeting the tumor. However, a study published in 2022 [216] demonstrated that an addition of the epidermal growth factor receptor (EGFR) inhibitor, gefitinib, alone or together with IFN treatment resulted in acquired resistance to VSV in two otherwise VSV-sensitive standard cell lines and primary glioma cultures. In addition, Her2 protein overexpression was observed on VSV-sensitive cell lines but not on VSV-resistant ones, which suggests that Her2 can potentially be used as a biomarker of tumor susceptibility to VSV therapy. And the main point of this study is that the combination of anti-EGFR therapy (gefitinib) with IFN-sensitive OVs is not effective for tumors with higher Her2 expression.

It should be noted that combination therapy with OVs is a common practice all over the world [217]. However, there are not many studies devoted to the study of the effects of combined treatment with VSV and chemotherapy drugs. Perhaps in the near future we will see an increasing interest in such research, because this approach is quite promising due to its effectiveness.

### *3.3 Combination with Immunotherapy*

Recent successes of cancer immunotherapeutics are hindered by immunologically ‘cold’ tumors that do not respond to treatment. Specifically, a wide variety of clinically approved immunotherapeutics, including chimeric antigen receptor (CAR)-T cells and ICI, are beneficial in only a small number of cancer patients, as a large subset of patients with immunologically ‘cold’ tumors respond poorly to the treatment [218,219]. Therefore, strategies that utilize OVs for ‘warming’ cold tumor microenvironments are attractive as they increase the effectiveness of ICIs. Multiple ongoing clinical trials evaluate the combination of VSV with ICIs. For example, CTLA-4 and PD-1, inhibitory receptors expressed on the surface of T cells, have become therapeutic targets for mitigation of the immunosuppressive tumor environment and promotion of antitumor immunity. Interaction of these inhibitory molecules with their corresponding ligands on tumor cells leads to T cell dysfunction and exhaustion [59].

Several preclinical studies relying on a combination of VSV and ICI have revealed promising candidates [220]. VSV expressing mIFN$\beta$ and NIS has been reported to yield significantly increased numbers of intratumoral $CD4^+$ and $CD8^+$ T lymphocytes when combined with an anti-PD-1 antibody as compared to VSV or anti-PD-1 therapy alone in a syngeneic model of acute myeloid leukemia. Furthermore, combination therapy with the anti-PD-1 antibody and VSV therapy led to significant tumor growth inhibition and an increase in survival compared to VSV monotherapy. Another approach to cancer treatment relies on the activation of NK cells, which play an important role in tumor immunosurveillance and anti-tumor immunity. Unlike traditional T cells that recognize peptide antigens in the context of major histocompatibility complex (MHC) I or II, NK cells recognize endogenous and exogenous glycolipids presented *via* the MHC I-like molecule CD1d [221,222]. For example, one of the studies [222] using mouse models of pancreatic cancer showed that NK activation in combination with VSV delivering IL-15 enhances the anti-tumor immune response against pancreatic cancer cells, leading to increased tumor regression and overall survival time. The authors also demonstrated that while anti-PD-1 monotherapy was ineffective, the addition of anti-PD-1 blockade to the combined immunotherapy further enhanced and prolonged the immune response, leading to extended tumor control and increased survival. IL-15 is a proinflammatory cytokine essential for the survival and function of many anti-tumor immune cells, including NK cells, and has been shown to increase immune targeting of cancer. Combining VSV delivering Smac (a pro-apoptotic protein) with the anti-PD-1 antibody greatly extended the survival of tumor-bearing mice, achieved a long-term survival of 44%, and altered the TME [223].

Collectively, these studies demonstrated that combinations of OVs with traditional radio-, chemo-, and im-

munotherapies offer an improved strategy for eliciting potent induction of the antitumor immune response by improving activation, recruitment, and infiltration of immune cells into tumor tissues. Preclinical studies have demonstrated that VSV is a promising OV; as a result, a human clinical trial using VSV is currently in progress.

## 4. Clinical Studies

The therapeutic effect of VSV monotherapy is still limited, and more focus is given to treatment strategies that combine OV therapy with other existing therapies. As of 2024, the total number of OV-based clinical trials registered on ClinicalTrials.gov reached 220. The majority of the clinical trials are phase I (n = 132). There are 42 additional clinical trials reported as phase I/II, 39 as phase II, 3 as phase III, 1 as phase II/III, and 3 as phase IV. Only 12 clinical trials, listed in Table 4, rely on VSV, however, it has a good safety profile, as has been proven by the active usage of the vaccine against the Ebola virus [224].

The OV-based therapeutic strategies include both OV monotherapies and their combinations with traditional and immunotherapies. The number of clinical trials has grown over the past 8 years, and this trend is expected to continue. Furthermore, preliminary data forecasts that OVs are likely to be an integral part of cancer immunotherapy in the near future. The majority of clinical trials based on VSV are combination therapies, and the most common modality administered in combination with an OV is ICI.

Only one study based on VSV (NCT02923466) was completed. Its main goal was to determine the safety profile after intratumoral (IT) or intravenous (IV) administration of a single VSV-IFN$\beta$-NIS dose or combined IT followed by IV VSV-IFN$\beta$-NIS, with or without IV administration of avelumab every two weeks, in patients with refractory advanced/metastatic solid tumors. Avelumab is a human monoclonal antibody (mAb) that binds to PD-L1 and prevents its interaction with the PD-1 receptor. While it is approved in several countries as monotherapy [225], it is important to mention that high expression levels of PD-L1 were detected around the primary tumor but not in distant metastases, leading to resistance to this immunotherapy [226,227]. VSV-IFN$\beta$-NIS is now in phase I/II trial (NCT03647163) in combination with pembrolizumab (an anti-PD1 antibody) in patients with non-small cell lung cancer and head and neck cancer and in combination with ipilimumab/nivolumab in patients with relapsed or refractory neuro-endocrine tumors. Current data suggests that VSV-IFN$\beta$-NIS doses of up to $1.7 \times 10^{11}$ TCID50 per patient are safe and likely more effective. VSV-hIFNbeta-NIS is also tested in combination with cyclophosphamide and ipilimumab in patients with relapsed or refractory multiple myeloma, acute myeloid leukaemia or lymphoma (NCT03017820). Cyclophosphamide is a nitrogen mustard drug that exerts its effects through the alkylation of DNA [228], while ipilimumab is a humanized monoclonal anti-CTLA-4 antibody approved by the Food and Drug Administration (FDA) in 2011 that increases the effect of antitumor therapy [229,230]. VSV-IFN$\beta$-NIS is also in a phase II trial (NCT04291105) in combination with cemiplimab (also an anti-PD1 antibody) in patients with colorectal, head and neck carcinoma, and melanoma. According to the study design, VSV-IFN$\beta$-NIS will be intratumorally administered on day 1 and every 3 weeks for as long as there is clinical benefit, while cemiplimab will be given intravenously. Other VSVs with similar insertions are also studied for their anti-cancer benefits (NCT03120624, NCT02923466), but these are currently in phase I, as is VSV used in combination with a PD-1 inhibitor (toripalimab) in patients with advanced malignant solid tumors (NCT05644509).

VSV-IFNbetaTYRP1, which includes human IFN$\beta$ for protection of healthy cells and TYRP1, expressed mainly in melanocytes and melanoma tumor cells, which may trigger a strong immune response aiming at melanoma tumor cells, is tested by intravenous and intratumoral injections in patients with previously treated metastatic melanoma (NCT03865212). The phase I trial studies the side effects and optimal dosage of VSV-IFNbetaTYRP1, while also gathering preliminary data on tumor response rate and progression-free survival time of VSV-IFNbetaTYRP1 therapy among patients. VSV-IFN-beta is tested in the phase I trial (NCT01628640) against refractory liver cancer or advanced solid tumors with lesions that have spread to other parts of the body and do not respond to treatment. One of the objectives is to estimate the tumor response rate, injected lesion and distant lesion necrosis rates, and overall survival. VSV-GP128, which carries the envelope glycoprotein of the visceral non-neurotropic WE-HPI strain of LCMV, is an integral part of the prime-boost regimen together with ATP128 (NCT04046445) in the phase I trial in patients with stage IV colorectal cancer.

## 5. Conclusions

VSV continues to be a very promising OV due to its many benefits: (i) absence of pathogenicity to humans; (ii) low immunity compared to other OVs, such as AdV; (iii) heightened sensitivity to the IFN response; (iv) cytoplasmic site of replication; (v) ability to replicate in hypoxic TME; (vi) inability to integrate into the host genome; and (vii) easily manipulated genome. The 12-hour lytic replication cycle leads to VSV's fast spread in tumor cells compared to DNA viruses. In addition, the high rate of replication and the ability to infect various cell lines allow for easy VSV production, an important factor for any drug development. The fact that VSV does not require specific receptors on the surface of cancer cells, unlike AdV [231], explains its wide tropism and the ability to exert its oncolytic action on a variety of cancer cells. VSV therapy can be administered both intratumorally and intravenously. VSV's systemic administration, possible due to the low prevalence of VSV-nAb in patients, can lead to the effective destruction of metastatic

tumor lesions. The problem may arise when the therapeutic regimen requires the administration of multiple doses, as viral G protein is capable of inducing a strong immune response, leading to the production of nAbs.

Despite all the listed benefits, it is important to remember that not all types of malignancies are susceptible to VSV-based therapy. For example, WT-VSV possesses neurotoxicity, which prevents its use for the treatment of central nervous system, brain, and neuroendocrine cancers. It is also important to get an in-depth understanding of VSV spread in the tumor and host, as safe virotherapy ultimately requires OV clearance from the body. Unfortunately, the same mechanisms can prematurely eliminate VSV before it completes the task.

Since the development of VSV as an OV, many novel approaches have evolved to create a safe and more efficient virus. These include, among other strategies discussed above: (i) introduction of mutations in G protein for better tropism; (ii) introduction of mutations in M protein to avoid off-target replication; (iii) addition of immunostimulatory factors for immune activation with the purpose of enhancing the anti-tumor response. Even though these VSV genome modifications led to increased targeting, efficacy, and safety of the virus, many aggressive cancer types, such as PDAC [232], head and neck cancer [233] and others, demonstrated resistance to VSV due to the absence of IFN-I signalling defects, among other factors. For this reason, combination therapy, which relies on two types of therapies for an enhanced synergistic effect, is an approach that gives hope for the treatment of tumors that are not destroyed by VSV or traditional therapy, such as RT, alone. The high toxicity and severe side effects of chemotherapy, for example, can be decreased as a result of a possible dose reduction due to its combination with VSV. Importantly, VSV's ability to replicate in hypoxic TME gives this therapy an advantage in combating tumors that are resistant to chemotherapy as a result of hypoxic conditions. Combination with immunotherapy should activate the recruitment of specific populations of immune cells to the tumor site and increase the natural anti-tumor response at the same time. Currently, the ongoing or recruiting VSV-based clinical trials study VSV monotherapy and its combination with immunotherapy against different types of cancer, and their results (hopefully positive) are eagerly anticipated.

Several important factors affect the outcome of the studies: cancer model, VSV phenotype, tumor size, viral dose, number of treatments, and administration route. As these vary greatly between studies, it is difficult to compare and draw meaningful conclusions. Each study's outcome is unique due to the above-mentioned factors. It is also important to remember the differences between various human and murine cancer models in terms of sensitivity to the IFN-I response, with the latter being more sensitive. This means that the outcome of a preclinical study will not necessarily translate into the success of a clinical study (and vice versa). Therefore, the future success of VSV oncotherapy is likely to be determined by the optimal choice of cancer models most susceptible to its action, improved modifications of the virus genome, optimal effective viral doses, enhanced immune response due to targeted delivery of specific immunostimulatory factors, and synergistic effects through combinatory therapeutic approaches.

## Abbreviations

4-1BBL, tumor necrosis factor ligand superfamily member 9; 5FC, 5-fluorocytosine; AAV, Adeno-associated virus; AdV, Adenovirus; APCs, antigen-presenting cells; CAR, chimeric antigen receptor; CCL, CC chemokine ligand; CD, cluster of differentiation; CD/UPRT, cytosine deaminase/uracylphosphoribosyltransferase; CEA, carcinoembryonic antigen; COCV, Cocal virus; CXCL, CXC chemokine ligand; DelNS1, Influenza A virus with deleted NS1 gene; EC50, semi-maximal effective dose; EGFR, epidermal growth factor receptor; FAST, small transmembrane proteins associated with reovirus fusion; G, glycoprotein; GCV, ganciclovir; GFP, green fluorescent protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; GP128, envelope glycoprotein of the visceral non-neurotropic WE-HPI strain of LCMV; GSDME, caspase-3/gasdermin E; H-1 PV, H-1 parvovirus; hDCT, human dopachrome tautomerase; HIV, human immunodeficiency viruses; HSV, Herpes simplex virus; HSV1-sr39tk, mutant herpes simplex virus thymidine kinase; HSV-TK, Herpes simplex virus thymidine kinase; hTERT, human telomerase reverse transcriptase; HTLV-1, T-cell human lymphotropic virus; IAV, Influenza A virus; IC, intracranial; ICD, immunogenic cell death; ICIs, immune checkpoint inhibitors; IFN, interferon; IH, *via* the hepatic artery; IL, interleukin; IV, intravenous, IT, inratumoral; L, large polymerase protein; LCL161, Smac mimetic compound and inhibitor of apoptosis antagonist; LCMV, lymphocytic choriomeningitis virus; Le, 47 nt-long leader sequence; Lip-MP, plasmid encoding gene of M, which was complexed with the DOTAP-cholesterol liposome; M, matrix protein; ΔM51 or dM51, M with deletion of amino acid in position 51 of matrix protein; MEF, mouse embryonic fibroblasts; MeVac, Measles Schwarz vaccine; MG1, Maraba MG1 virus; MHC, major histocompatibility complex; MORV, Morreton virus; MV, measles virus; N, nucleocapsid protein; NDV, Newcastle disease virus; NIS, sodium iodide symporter; NKs, natural killer cells; OVs, oncolytic viruses; OX40L, tumor necrosis factor receptor superfamily member 4 (TNFRSF4) ligand; P, phosphoprotein; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed death-ligand 1; PET, positron emission; PFU, plaque-forming unit; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog; RdRp, RNA-dependent RNA polymerase; RNP, ribonucleoprotein complex; RT, radiotherapy; SFV, Semliki Forest virus; Sin, Sindbis virus; Sindbis GP, Sindbis

virus glycoprotein; Smac, second mitochondria-derived activator of caspases; SVA, Senecavirus A; TAAs, tumor-associated antigens; TCID50, 50% tissue culture infectious dose; TME, tumor microenvironment; TNF, tumor necrosis factor; Tr, 5′-terminal sequence; TRAIL, TNF-related apoptosis-inducing ligand; TYRP1, tyrosinase related protein 1; UPRT, uracil phosphoribosyltransferase; VEGF, vascular endothelial growth factor; VEGI, vascular endothelial growth inhibitor; VG9, vaccinia virus train Guang9; VSAV, Vesicular stomatitis Alagoas virus; VSIV, Vesicular stomatitis Indiana virus; VSNJV, Vesicular stomatitis New Jersey virus; VSV, Vesicular stomatitis virus; VV, vaccinia virus; WT-VSV, wild-type VSV.

## Author Contributions

EM and AK conceptualized. MZ and AR analysed the data. MZ, AR, and EM wrote the manuscript. EM provided help and advice. AK provided financial support for the project. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.


## Acknowledgment

Not applicable.

## Funding

This work was supported by the grant of the state program of the «Sirius» Federal Territory «Scientific and technological development of the «Sirius» Federal Territory» (Agreement 18-03).


## Conflict of Interest

The authors declare no conflict of interest.

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