

# Recent advances in vesicular stomatitis virus-based oncolytic virotherapy: a 5-year update

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## Abstract

Oncolytic virus (OV) therapy is an anti-cancer approach that uses viruses that preferentially infect, replicate in and kill cancer cells. Vesicular stomatitis virus (VSV, a rhabdovirus) is an OV that is currently being tested in the USA in several phase I clinical trials against different malignancies. Several factors make VSV a promising OV: lack of pre-existing human immunity against VSV, a small and easy to manipulate genome, cytoplasmic replication without risk of host cell transformation, independence of cell cycle and rapid growth to high titres in a broad range of cell lines facilitating large-scale virus production. While significant advances have been made in VSV-based OV therapy, room for improvement remains. Here we review recent studies (published in the last 5 years) that address 'old' and 'new' challenges of VSV-based OV therapy. These studies focused on improving VSV safety, oncoselectivity and oncotoxicity; breaking resistance of some cancers to VSV; preventing premature clearance of VSV; and stimulating tumour-specific immunity. Many of these approaches were based on combining VSV with other therapeutics. This review also discusses another rhabdovirus closely related to VSV, Maraba virus, which is currently being tested in Canada in phase I/II clinical trials.

## INTRODUCTION

Oncolytic virus (OV) therapy is an emerging anti-cancer approach that uses viruses that preferentially infect, replicate in and kill cancer cells. Numerous pre-clinical and clinical successes have been reported [1, 2], and currently three OVs are approved for clinical use: herpes simplex virus 1 (HSV-1)-based T-VEC for melanoma, approved in the USA and later in the European Union [3]; enteric cytopathic human orphan virus 7-based RIGVIR for melanoma, approved in Latvia, Georgia and Armenia [4]; and adenovirus type 5-based Gendicine and Oncorine for head and neck squamous cell carcinoma in China [5].

Vesicular stomatitis virus (VSV) is a prototypic non-segmented negative-stranded RNA virus (order *Mononegavirales*, family *Rhabdoviridae*, genus *Vesiculovirus*) [6]. The small 11 kb genome of VSV encodes five proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large polymerase protein (L) [6]. Horses, cattle, pigs and a range of other mammals and their insect vectors are natural hosts of WT VSV. WT VSV

infections of livestock are non-lethal and cause fever and blister-like lesions on the oral cavity, feet and teats, and human VSV infections are generally asymptomatic and limited to agricultural and laboratory workers [6]. Human infections with WT VSV are relatively common throughout the tropical Americas, however only one published report described VSV-mediated non-lethal encephalitis in a 3-year-old Panamanian boy [7].

Several factors make VSV a promising OV: lack of pre-existing human immunity against VSV, a small and easy to manipulate genome, cytoplasmic replication without risk of host cell transformation, independence of cell cycle and rapid growth to high titres in a broad range of cell lines allowing large-scale production of virus [8, 9]. One of the distinctive features of VSV is its pantropism. Ubiquitously expressed cell-surface molecules such as the low-density lipoprotein receptor, phosphatidylserine, sialoglycolipids and heparan sulfate have all been shown to be utilized by VSV for cell attachment [9]. While such pantropism does not allow VSV to distinguish non-malignant ('normal') cells

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**Abbreviations:** AAV, adeno-associated virus; AML, acute myeloid leukemia; F, fusion protein; G, glycoprotein; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; H, haemagglutinin protein; HCV, hepatitis C virus; HDAC, histone deacetylase; HPV, human papillomavirus; HSV-1, herpes simplex virus 1; IKK, I $\kappa$ B kinase; IRES, internal ribosome entry site; ISG, IFN-stimulated gene; i.t., intratumoural; i.v., intravenous; L, large polymerase protein; LCMV, lymphocytic choriomeningitis virus; M, matrix protein; MDSC, myeloid-derived suppressor cell; MUC1, mucin 1 protein; MV, measles virus; N, nucleocapsid protein; nAb, neutralizing antibody; NF- $\kappa$ B, nuclear factor-kappa B; NIS, sodium iodide symporter; OV, oncolytic virus; P, phosphoprotein; PD-1, programmed death 1; PD-L1, programmed death ligand 1; PDAC, pancreatic ductal adenocarcinoma; PEG, polyethylene glycol; TAA, tumour-associated antigen; RT, radiation therapy; VLV, virus-like vesicle; VSV, vesicular stomatitis virus; VV, vaccinia virus.

from cancer cells based on their differential receptor expression profiles, the relative independence of VSV from a single receptor can be an advantage, allowing VSV-based OV to target a wide range of tumour types. In contrast, other OVs could be limited by the expression of their receptor, such as adenovirus 5-based OVs that require the coxsackievirus and adenovirus receptor for efficient attachment to the target cells [10].

Oncoselectivity of VSV is generally based on the lower type I IFN-associated antiviral potential of cancer cells compared to normal cells [8, 9]. Most tumours have defective or inhibited type I IFN signalling [11], likely because many IFN responses are anti-proliferative, anti-angiogenic and pro-apoptotic [12]. As WT VSV is sensitive to type I IFN responses, it preferentially replicates in cancer cells. However, such inherent oncoselectivity of WT VSV is not sufficient, as it is able to inhibit type I IFN signalling through one of the functions of the VSV M protein. This multifunctional viral protein can localize to the nuclear envelope and inhibit nucleocytoplasmic trafficking of cellular mRNAs, thus impeding antiviral gene expression in infected cells, including normal cells [13]. As a result, WT VSV can exhibit unacceptable toxicity, most notably neurotoxicity. In rodent models, VSV can cause neurotoxicity when administered intracranially [14, 15], intranasally [16], intravascularly [17] and intraperitoneally [18]. In non-human primates, an intrathalamic administration results in severe neurological disease [19]. To address this important safety issue, various recombinant VSVs have been generated with improved safety and oncoselectivity profile [8]. For example, VSV M51 M mutants have a mutation or deletion of the methionine residue at position 51 of the M protein. This mutation prevents the M protein from binding to the Rae1-Nup98 mRNA export complex required for cellular mRNA (including mRNAs for antiviral genes) transport and subsequent translation. As a consequence, VSV- $\Delta$ M51 is not able to inhibit antiviral responses in initially infected cells (normal or cancer), which limits its replication in the neighbouring normal cells but not in cancer cells as they are typically defective in antiviral responses [20, 21]. Another common approach is to use VSV-encoding IFN $\beta$  (VSV-IFN $\beta$ ), which oncoselectivity is based on virus-encoded IFN $\beta$  expression that stimulates an innate immune response in normal cells but not in type I IFN defective cancer cells [22, 23]. IFN $\beta$  also stimulates tumour-specific immunity [24]. VSV-IFN $\beta$  showed no signs of neurotoxicity at any time point in rhesus macaques when administered via intrahepatic injection [22]. As a result, VSV-IFN $\beta$ -sodium iodide symporter (VSV-IFN $\beta$ -NIS) (encodes NIS in addition to IFN $\beta$ ) is currently being tested in the USA in several phase I clinical trials (see details at ClinicalTrials.gov for trials NCT02923466, NCT03120624 and NCT03017820).

While significant advances have been made in the use of VSV as an OV, room for improvement remains. In our previous comprehensive review of VSV-based oncolytic virotherapy published in 2012 [8], we highlighted successes and

discussed different approaches that have been shown to overcome the challenges of VSV-based OV therapy. Those approaches focused on improving VSV oncoselectivity, safety and oncotoxicity; preventing premature clearance of VSV; and inducing/stimulating tumour-specific immunity. Here we review novel approaches published in the last 5 years, which have addressed the ‘old’ and ‘new’ challenges of VSV-based OV therapy. We also discuss another rhabdovirus closely related to VSV, Maraba virus, which is currently being tested in Canada in phase I/II clinical trials (see details at ClinicalTrials.gov for trials NCT02285816 and NCT02879760).

We focus here mainly on articles published in the last 5 years. For older publications, please refer to our previous review [8], as well as other reviews describing VSV as an OV [25, 26]. Newly developed VSV recombinants described in the last 5 years are summarized in Table 1 and new VSV/drug combinations are summarized in Table 2.

## IMPROVING ONCOSELECTIVITY AND SAFETY

In our previous review, we identified eight major approaches which had been shown to improve VSV oncoselectivity (OV ability to preferentially replicate in and destroy cancerous cells over non-cancerous cells) and safety without compromising its oncolytic abilities [8]: (i) using VSV encoding mutant M protein unable to inhibit antiviral response in normal cells; (ii) using VSV encoding IFN $\beta$  to attenuate viral replication in normal tissues and stimulate antitumour immunity; (iii) attenuation of VSV through disruption of normal gene order; (iv) mutating the VSV G protein to limit/direct VSV tropism; (v) introducing targets for microRNA expressed in normal cells into the VSV genome to inhibit VSV-associated toxicities; (vi) pseudotyping VSV to inhibit VSV neurotropism; (vii) experimental adaptation of VSV to cancer cells; and (viii) using semi-replicative VSV [8]. Here we will provide updates on some of these approaches and discuss new ones.

### Using VSVs encoding type I and type III IFNs

Several recent pre-clinical studies have shown that VSV-IFN $\beta$  and VSV-IFN $\beta$ -NIS (additionally expresses the NIS to track virus spread) are oncoselective and safe in a variety of tumour and animal models [24, 27–31]. Although mice and rats continue to serve as the most commonly used animal models for VSV-based OV therapy, a recent study evaluated the safety of intravenously administered VSV-IFN $\beta$ -NIS in purpose-bred beagle dogs. The data indicated that an intravenous (i.v.) dose of  $10^{10}$  TCID<sub>50</sub> was well-tolerated by dogs. Furthermore, no infectious virus was detectable in plasma, urine or buccal swabs at any tested doses [29]. Importantly, VSV-IFN $\beta$ -NIS is currently in several phase I clinical trials in the USA: against refractory solid tumours (see details at ClinicalTrials.gov trial NCT02923466), stage IV or recurrent endometrial cancer (trial NCT03120624), and relapsed or refractory multiple myeloma, acute myeloid leukemia or T-cell lymphoma (trial NCT03017820).

**Table 1.** Novel VSV recombinants used as oncolytic agents against cancer (reported 2012–2017)  
 VSV recombinants used as oncolytic agents against cancer designed before 2012 can be found in Table 1 of our previous review [8]. \* Designed to prevent premature clearance of VSV.

Novel oncolytic VSV	Virus description	Ref	Designed to improve:				Tumour immunity
			Oncoselectivity	Safety	Direct oncototoxicity	VSV survival*	
<b>WT and miscellaneous</b>							
VSV-12/GFP	VSV expressing GFP reporter gene at position 1 and 2. Attenuated because all VSV genes are moved downward, to positions 3–7. Safe and still effective as an OV.	[36]		X			
VSV <sub>FMDV</sub> and VSV <sub>HRV</sub>	Insertion of foot-and-mouth disease virus IRES and human rhinovirus type 2 IRES elements before the start codon of the M gene. Controlled the translation of VSV-M.	[55]	X	X			
<b>Foreign glycoprotein</b>							
VSV- $\alpha$ RGD, - $\alpha$ Echi, - $\alpha$ HER2 and - $\alpha$ EGFR	VSV encoding a modified VSV-G displaying tumour vasculature-targeting ligands (cyclic RGD and echistatin) and single-chain antibodies (scFv) against tumour-specific antigens (human epidermal growth factor receptor-2 and epidermal growth factor receptor)	[37]	X	X			
VSVFH with H mutations	Chimeric VSV (lacks VSV-G) encoding the MV F and H. Different mutations were made to H to disrupt attachment to nectin-4, SLAM and CD46.	[44]	X	X			
VSVFH- $\alpha$ HER2	Chimeric VSV (lacks VSV-G) encoding the MV F and H displaying single-chain antibodies (scFv) specific for human epidermal growth factor receptor-2.	[42]	X	X			
VSV-CD133	Retargeted VSV to cells that expressed the targeted receptor.	[43]	X	X			
rVSV(GP)	Chimeric VSV (lacks VSV-G) encoding the MV F and H displaying single-chain antibodies (scFv) specific for CD133. Retargeted VSV to cells that expressed the targeted receptor.	[46]	X	X		X	
VSV-gp160G	Chimeric VSV (lacks VSV-G) encoding the non-neurotropic glycoprotein of LMCV.	[47]	X	X			
VSV-RABV-G, VSV-LASV-GPC, VSV-LCMV-GPC, VSV-EBOV-GP, VSV-MARV-GP	Chimeric VSV (lacks full-length VSV-G) encoding a hybrid fusion protein, combining domains from gp160 of HIV-1 and VSV-G. Retargeted VSV to human T-cell lymphotropic virus type 1-associated adult T-cell leukemia.	[48]	X	X			
VSVAG-CHICKV, VSVAG-H5N1, VSVAG-Nipah F, VSVAG-Nipah G, VLV	Chimeric VSV (lacks VSV-G) encoding the G of rabies virus, Lassa virus, LCMV, Ebola virus or Marburg virus.	[49]	X	X			
VSV/Maraba G	Chimeric VSV (lacks VSV-G) encoding the G of chikungunya virus, influenza virus H5N1, and Nipah virus F and G. For VLV only VSV-G is used and all other VSV genes are deleted and replaced by the Semliki Forest virus non-structural protein genes.	[112]				X	
<b>Cancer suppressors</b>	VSV-G is deleted and replaced by the glycoprotein genes from Maraba virus. Resistant to non-immune human serum.						
VSVAM51eqFP650-p53wt, -p53CC, -p53CC/fs	VSV expressing the human p53 gene.	[98]	X	X	X		X
<b>Immunomodulation</b>							
VSV-mIFNbeta-hIFNbeta-NIS	VSV expressing the murine (m) or human (h) IFN $\beta$ gene and the thyroidal NIS.	[31]	X	X	X		X
VSV28.1 and VSV28.2	VSV expressing IL-28, a member of the type III IFN (IFN-A) family, at position 1 and 5.	[34]	X	X			X
VSVA51-IFN $\gamma$	VSV expressing the murine IFN- $\gamma$ gene.	[116]	X	X	X		X
VSV-NRAS, VSV-TYRP1 and VSV-CYC1	VSV expressing neuroblastoma-Ras, cytochrome c and tyrosinase-related protein 1.	[117]	X	X			X
VSV-HIF-2 $\alpha$ , VSV-Sox-10, and VSV-c-Myc	VSV expressing hypoxia-inducible factor [HIF]-2 $\alpha$ , Sox-10, c-Myc.	[118]	X	X			X

**Table 2.** Novel combination of VSV with other drugs against cancer (reported 2012–2017)

↑increase; ↓decrease.

VSV variant	Drug name	Drug type	Ref	Designed to improve:		Observed <i>in vitro</i> effects		Observed <i>in vivo</i> effects
				Oncoselectivity	Safety	Direct Oncotoxicity		
VSV-ΔM51-GFP	gemcitabine	Antimetabolite antineoplastic agent	[54]			X		↓ tumour volume in mouse PDAC xenografts
VSV-CT9-M51 and VSV-rp30	IFN-αA/D, ribavirin and chloroquine	IFN, viral nucleoside inhibitor and quinolone respectively	[56]		X		↓ replication, cytotoxicity and infectivity in neuronal cell cultures	↓ neurotoxicity and ↑ survival after intracranial VSV-CT9-M51 injection in Swiss-Webster mice
VSV-GFP	NSC74859	STAT3 inhibitor	[57]	X	X		↓ HCC cell proliferation	↓ toxicity and ↑ maximum tolerated dose of VSV in C57BL/6 mice
VSV-ΔM51-GFP and VSV-GFP	JAK Inhibitor I	pan-JAK inhibitor	[64, 66, 68]			X	↓ replication in primary human hepatocytes ↓ cytotoxicity in primary rat neurons and glial cells ↓ MX1 and OAS expression in resistant PDAC cell lines ↑ replication and oncolysis in resistant PDAC cell lines	
VSV-ΔM51-GFP, VSV-GFP and VSV-GP	ruxolitinib	JAK1/JAK2 inhibitor	[66–69]			X	↓ IRF-9, IRF-7 and OAS expression in human head and neck cancer cells ↑ infection, spread and progeny yield in human head and neck cancer cells ↓ MX1, EPST11, XAF1 and GBP1 expression in PDAC cell lines ↑ replication and oncolysis in resistant PDAC cell lines ↓ IRF-9, IRF-7 and OAS in human head and neck cancer cells ↑ infection, spread and progeny yield in human head and neck cancer cells ↑ replication and oncolysis in ovarian cancer cell lines	↓ tumour volume and ↑ survival in both subcutaneous and orthotopic xenograft mouse ovarian cancer models
VSV-ΔM51-GFP and VSV	TPCA-1	IKK-β inhibitor, but we demonstrated that it also inhibits JAK1	[66, 67, 71]			X	↓ MX1, EPST11, XAF1 and GBP1 expression in PDAC cell lines ↑ replication and oncolysis in resistant PDAC cell lines	
VSV, VSV-GFP and VSV-mIFN-NIS	BMS-345541	IKK inhibitor	[71, 72]			X	↓ MX1 and GBP1 in human glioma cells ↑ replication and oncolysis in human glioma cells but not in myeloma cells	
VSV-GFP and VSV-mIFN-NIS	bortezomib	proteasome inhibitor	[72]			X	↓ replication and oncolysis in myeloma cells	↓ tumour volume and ↑ survival in myeloma mouse model
VSV	Vorinostat	HDAC inhibitor	[73]			X	↑ NF-κB signalling ↓ IFN signalling	

Table 2. cont.

VSV variant	Drug name	Drug type	Ref	Designed to improve:			Observed <i>in vitro</i> effects	Observed <i>in vivo</i> effects
				Oncoselectivity	Safety	Direct Oncotoxicity		
wt-GFP and rM51R-GFP	curcumin	Natural agent	[75]			X	<ul style="list-style-type: none"> <li>↑ autophagy</li> <li>↑ replication</li> <li>↑ oncolysis in prostate cancer cell lines</li> <li>↓ NF-κB phosphorylation</li> <li>↓ STAT1 activation</li> <li>↓ Bcl-xl expression</li> <li>↑ infection</li> </ul>	<ul style="list-style-type: none"> <li>↓ tumour volume of prostate tumours in BALB/c nude mice</li> </ul>
VSV-Δ51-GFP and VSV-Δ51-Luc	triptolide	Natural agent	[76]			X	<ul style="list-style-type: none"> <li>↑ oncolysis in prostate cancer cell lines</li> <li>↑ replication in human prostate cancer cell lines and human B-lymphoma cell line</li> <li>↓ type I IFN signalling</li> <li>↑ apoptosis</li> </ul>	<ul style="list-style-type: none"> <li>↑ replication in a prostate mouse xenograft model</li> <li>↓ tumour volume and survival in mammary adenocarcinoma mouse xenograft model</li> </ul>
VSVΔ51	colchicine	microtubule-destabilizing agent	[77]			X	<ul style="list-style-type: none"> <li>↓ type I IFN production</li> <li>↑ viral spread</li> <li>↑ infection-induced cytokines</li> <li>↑ viral bystander effects</li> </ul>	<ul style="list-style-type: none"> <li>↑ spread and oncolytic activity in colon cancer nude mouse model and breast cancer syngeneic mouse model</li> </ul>
VSV-ΔM51	Polybrene or DEAE-dextran and ruxolitinib	Polycations and JAK1/JAK2 inhibitor	[85]			X	<ul style="list-style-type: none"> <li>↑ attachment</li> <li>↑ replication</li> <li>↑ oncolysis of PDAC cell lines</li> </ul>	
VSVM51R	Obatoclax or ABT-737	BCL-2 inhibitors	[89]			X	<ul style="list-style-type: none"> <li>↑ apoptosis</li> <li>↑ autophagy</li> <li>↑ oncolysis</li> </ul>	<ul style="list-style-type: none"> <li>↓ tumour volume and survival of chronic lymphocytic leukemia xenograft mouse model</li> </ul>
VSVΔ51-GFP	LCL161	SMAC mimetic inhibitor	[90]			X	<ul style="list-style-type: none"> <li>↑ oncolysis but no synergy with VSV</li> </ul>	<ul style="list-style-type: none"> <li>↓ tumour volume and survival in rhabdomyosarcoma syngeneic mice</li> </ul>
VSVM51R	sumitinib	Anti-angiogenic (inhibitor of VEGF-R and PDGF-R)	[102]			X	<ul style="list-style-type: none"> <li>↓ eIF2-α phosphorylation (substrate of PKR)</li> <li>↑ replication</li> <li>↑ oncolysis</li> </ul>	<ul style="list-style-type: none"> <li>↓ tumour volume of prostate tumours in nude mice, breast tumours in immunocompetent mice and renal cell carcinoma tumours in nude mice</li> </ul>
VSVΔ51	ZD6126	Vascular disrupting agent	[99]			X	<ul style="list-style-type: none"> <li>↑ oncolysis</li> </ul>	<ul style="list-style-type: none"> <li>↑ survival of hypopharyngeal squamous cell carcinoma mouse xenograft model</li> </ul>

Other antiviral IFNs have also been evaluated in combination with VSV. A recent study showed that IFN- $\alpha$ 2a was significantly less effective in protecting head and neck cancer cells when compared to IFN $\beta$ , whereas normal cells were equivalently protected by both IFNs. This result suggests that oncoselectivity may be enhanced by pairing VSV with IFN- $\alpha$ 2a rather than IFN $\beta$ . This difference could be due to the threshold for induction of type I IFN signalling being raised in some cancer cells compared to normal cells (e.g. receptor levels, negative regulatory factors) and to IFN $\beta$  binding with 1000-fold higher affinity to IFNAR than IFN- $\alpha$ 2a [32]. Previously, cellular IFN- $\lambda$  (type III IFN) produced during VSV infection was shown to enhance VSV therapy in the B16ova/C57Bl/6 mouse model by activating NK cells against B16ova cells [33]. Recently, two VSV vectors encoding functionally active IFN- $\lambda$ , VSV28.1 and VSV28.5, were generated [34]. VSV28.1 expresses IFN- $\lambda$  at position 1 and VSV28.5 expresses IFN- $\lambda$  at position 5. Both VSV vectors were attenuated in IFN- $\lambda$  responsive non-malignant MMDH3 immortalized mouse hepatocytes. *In vivo*, VSV28.1 showed reduced replication and spread in the lung and spleen of BALB/c mice following intranasal infection while still inducing similar CD8 T-cells and antibody responses when compared to non-attenuated VSV [34].

#### Attenuation of VSV through disruption of normal gene order

Previously, to attenuate VSV, a reporter gene was encoded on the 3' end of the VSV genome in the first genomic position to shift the N-P-M-G-L genes from positions 1 to 5 to positions 2 to 6 and thus reduce expression of VSV genes [35]. More recently, to attenuate VSV even more, two reporter genes were added to shift VSV genes from positions 1 to 5 to positions 3 to 7. This newly attenuated virus (VSV-12'GFP) showed smaller plaques and slower growth kinetics. Even though less replication was seen in human glioma and melanoma cells due to attenuation almost no virus was detected in normal cells hence improving VSV safety. *In vivo*, i.v. administration of VSV-12'GFP targeted brain tumours and no infection was found in normal tissue surrounding the tumours. Injecting VSV-12'GFP intratumourally into subcutaneous human rU-87 tumours of SCID mice suppressed the growth of the tumours and enhanced the survival of mice [36].

#### Mutating the VSV G protein to limit/direct VSV tropism

VSV oncoselectivity can also be improved through generation of chimeric VSV recombinants encoding a modified VSV G protein (VSV-G) or heterogeneous glycoproteins instead of the VSV G. In a proof of principle study, VSV was retargeted by engineering VSV-G to display tumour-targeting ligands. The 49-amino acid echistatin domain could be inserted at two sites on VSV-G. VSV-echistatin was able to specifically target integrin  $\alpha$ v $\beta$ 3 *in vitro* and showed equal oncolytic efficacy in a mouse myeloma model [37]. The same study also showed that larger polypeptide ligands like single-chain antibodies with specificity to

tumour-associated receptors EGFR and HER2 could be inserted at the N terminus of the G protein to target cancer cells more accurately [37].

#### Pseudotyped and chimeric VSVs to inhibit VSV neurotropism

Measles virus (MV) targets CD46 (overexpressed in most cancers) and kills infected cells by inducing fusion of infected cells with uninfected neighbours, but it propagates slowly [38]. VSV spreads rapidly, directly lysing tumour cells primarily via apoptosis induction, but it can be neurotoxic [8]. Chimeric VSV recombinant VSV-FH encoding MV fusion (F) and haemagglutinin (H) glycoproteins instead of VSV-G demonstrated diminished neurotoxicity, selectively targeted CD46 on tumour cells, replicated rapidly with VSV kinetics and combined the tumour-killing mechanisms of both viruses. VSV-FH was superior to MV and VSV- $\Delta$ M51 in a myeloma xenograft model [39, 40]. However, VSV-FH can be neurotoxic when given intravenously to CD46 transgenic mice lacking a functional type I IFN receptor [40]. To reduce the neurotoxicity of VSV-FH, retargeted VSV-FH vectors were generated displaying single-chain antibodies (displayed as C-terminal extensions on the H protein of MV) with specificity to tumour-associated receptors. Previously, using this approach, replication-deficient pseudotyped VSV-FH particles (were generated using VSV- $\Delta$ G and a packaging cell line expressing MV F and H) were used to retarget VSV to cancer cells expressing epidermal growth factor receptor, folate receptor or prostate membrane-specific antigen in human tumour xenografts in mice [41]. More recently, a similar approach was used to retarget replication-competent chimeric VSV-FH to cancer cells, especially ovarian cancer cells, expressing HER-2/neu receptor [42]. In another study, VSV encoding CD133-targeted MV-H and the MV-F was generated and successfully targeted cancer cells expressing a putative marker of cancer stem cells CD133 in a subcutaneous hepatocellular carcinoma (HCC) xenograft model [43]. As MV also infects nectin4-positive airway epithelial cells, a modified VSV-FH recombinant was generated with H mutations that disrupts binding to nectin4, which were expected to improve the virus oncoselectivity by limiting its binding only to CD46. Unfortunately, disruption of virus binding to nectin4 also compromised CD46 binding because nectin4 and CD46 have substantially overlapping receptor-binding surfaces on H [44].

Previously, replication-deficient VSV (called VSV-GP), which is VSV pseudotyped with the non-neurotropic envelope glycoprotein of lymphocytic choriomeningitis virus (LCMV) instead of the VSV G protein, showed enhanced infectivity against malignant glioma cells while not harming primary neurons [45]. Recently, replication-competent VSV-GP was generated encoding LCMV glycoprotein instead of VSV-G, and it was shown that VSV-GP also has the ability of escaping humoral immunity by not eliciting a neutralizing antibody (nAb) response and thus allowing repeated systemic OV treatment without a loss of

therapeutic efficacy [46]. In another study, VSV was retargeted to aggressive malignant CD4/CD25<sup>+</sup> T lymphocytes [adult T-cell leukemia/lymphoma (ATL)] by replacing VSV-G with a hybrid fusion protein containing extracellular and transmembrane domains from HIV1 gp160 and the cytoplasmic region of VSV-G. VSV-gp160G showed no signs of neurotoxicity in immunodeficient mice and improved tumour burden relief in ATL-bearing mice [47]. VSV-G was also replaced by the glycoprotein from Ebola virus, Lassa virus, LCMV, rabies virus and Marburg virus in attempt to reduce neurotoxicity. VSV-LASV-GPC showed the most promising results by exhibiting no adverse side effects even when injected directly into the brain, and targeted and destroyed glioblastoma and melanoma [48]. VSV encoding glycoprotein genes from Nipah, chikungunya (CHIKV) and H5N1 influenza viruses also have been generated and tested. VSV $\Delta$ G-CHIKV showed substantially attenuated neurotropism and was safe in the healthy adult mouse brain. VSV $\Delta$ G-H5N1 was also safe in the adult brain but lethal in the young brain. Interestingly, although VSV $\Delta$ G Nipah F and VSV $\Delta$ G Nipah G did not show any adverse effects when used separately (due to the inability of each individual virus to effectively spread), the combination of these two viruses was even more neurotropic than WT VSV leading to death in the adult mouse brain [49]. In the same study, replication-competent virus-like vesicles (VLVs) were generated and tested, in which only the VSV glycoprotein gene was used and all other VSV genes were deleted and replaced by the Semliki Forest virus non-structural protein genes. VLV showed substantially attenuated neurotropism and was safe in the healthy adult mouse brain. This suggests that although VSV-G has been shown to be neurotropic and therefore problematic in the brain during VSV infection, G alone is not a neurovirulence factor in the absence of other VSV proteins and VSV replication [49].

### Experimental adaptation of VSV to cancer cells

VSV recombinants with enhanced oncoselectivity could be generated via directed experimental evolution (using serial passages) to adapt viruses to cancer cells. In a recent study, VSV was adapted to cells deficient for the tumour suppressor gene TP53 (defective WT p53 function is a hallmark of cancer cells) [50]. It was observed that some evolved VSVs showed increased fitness and cytotoxicity in p53<sup>-/-</sup> cells but not in the isogenic p53<sup>+/+</sup> cells. One of the evolved VSV lines significantly delayed tumour growth in mice compared to parental virus or untreated controls [50]. In another study, VSV- $\Delta$ M51 was passaged in normal type I IFN-secreting cells to determine the role of IFN in VSV- $\Delta$ M51 evolution. A total of 20 passages led to a modest recovery of IFN blocking capacity and to slight increases in viral fitness [51]. The WT M sequence was not restored but  $\Delta$ M51 mutation was instead compensated by changes in the VSV P protein. This indicates that, while the  $\Delta$ M51 mutation is stable, VSV- $\Delta$ M51 may recover its IFN suppression capacity after extended replication.

### Using non-replicative VSV

In severely immunosuppressed cancer patients, the potential for uncontrolled VSV spread may compromise the patient's safety. Previously, non-replicative VSV\* $\Delta$ G and VSV $\Delta$ L-dsRed were generated and, when combined, were as potent as WT VSV *in vitro* and induced long-term glioblastoma tumour regression in mice *in vivo* without neurotoxicity [52]. More recently, VSV was exposed to a wide range of UV irradiation intensities to generate non-replicating VSV particles [53]. When UV irradiated at a low dose, VSV lost its ability to replicate but, surprisingly, maintained potent cytotoxicity in Vero cells. Importantly, these replication-deficient VSV particles specifically targeted leukemic L1210 cancer cells and eradicated acute leukemia in a mouse model. Although the exact mechanism of oncoselectivity of such non-replicating VSV particles is unclear, the study showed that, compared to normal cells, the cancer cells were more sensitive to replication-deficient VSV-mediated cell death due to their defective type I IFN signalling [53]. In contrast to this study, one of our own studies showed that UV-killed VSV- $\Delta$ M51-GFP enhanced tumour growth in an immunocompetent mouse model of pancreatic ductal adenocarcinoma (PDAC) [54]. The mechanism is unclear and will be examined in our future studies.

### Other approaches to improve VSV oncoselectivity

In addition to the listed major approaches, there are also new methodologies that have been developed in the last 5 years to improve VSV oncoselectivity and safety. It has been discovered that some picornaviral internal ribosome entry site (IRES) elements possess restricted activity in neuronal tissues. As a consequence, they were engineered into VSV in an attempt to attenuate its neurotoxicity. IRES elements from human rhinovirus type 2 (HRV2) and foot-and-mouth disease virus (FMDV) were used to control the translation of the VSV M mRNA. IRES elements of HRV2 and FMDV severely attenuated neurotoxicity of VSV without perturbing its oncolytic potency in BALB/c mice bearing subcutaneous mouse plasmacytoma [55]. Another study evaluated the ability of 12 antiviral compounds and an adeno-associated virus (AAV) vector expressing murine IFN (AAV-IFN) in combination with VSV-CT9-M51 (VSV mutant that has the cytoplasmic tail of G truncated by removal of residues 9–29 and M51 mutation in M) and VSV-rp30 (generated by passaging VSV-G/GFP 30 times on glioblastoma cells) to control neuronal infection. A combination of AAV-IFN $\beta$  together with a broad spectrum antiviral drug ribavirin appeared to be the most effective combination in blocking VSV infection of neurons *in vitro*. *In vivo*, AAV-mIFN $\beta$  was the most effective by inhibiting VSV-CT9-M51 neurotoxicity in immunocompetent mice and increasing survival in VSV-CT9-M51 treated human glioblastoma-bearing immunodeficient mice. The study was more focused on safety, however some surviving mice showed complete tumour regression [56]. VSV has also been combined with NSC74859 (S3I-201), a specific inhibitor of STAT3 [57]. STAT3 overexpression in cancer cells

can lead to upregulation of genes encoding for apoptosis inhibitors, cell-cycle regulators and inducers of angiogenesis [58]. NSC74859 inhibited HCC cell growth *in vitro* and improved VSV oncoselectivity. Inhibition of STAT3 did not affect VSV replication in HCC cells, but decreased VSV replication in primary human hepatocytes. The mechanism of oncoselectivity is not yet understood but it appears to be IFN-independent. NSC74859 also protected primary rat neurons and glial cells from VSV cytotoxicity and reduced VSV toxicity in C57BL/6 mice. This combination allowed a significant increase in the maximum tolerated dose of VSV [57].

To further improve the safety of VSV-based OV therapy, it is important to understand host factors determining safety risks associated with VSV therapy. In a recent study, WT mice were challenged with VSV and produced IL-1 $\beta$  locally and systemically. Accumulation of IL-1 $\beta$  correlated with acute pathology (weight loss and fever) in mice. IL-1R $^{-/-}$  mice were protected from acute weight loss after VSV challenge, controlled VSV replication, had strong humoral and cellular immune responses, and were immune to rechallenge with VSV. These results suggest that VSV vectors engineered to suppress the induction of IL-1 $\beta$  or signalling through the IL-1R would be safer [59]. In another study, mice bearing systemic 5TGM1 myeloma tumour cells were treated with VSV-IFN $\beta$  intravenously and developed meningoencephalitis. Histological analysis revealed that systemically administered 5TGM1 cells seed to the CNS, forming meningeal tumour deposits, and that VSV infects and destroys these tumours. However, tumour destruction was accompanied by meningeal damage due to direct transmission of virus to adjacent neural tissue. This study suggest that more safety measures have to be taken when clinical testing of VSV-IFN $\beta$  is done on patients with meningeal tumour deposits [60]. In general, the choice of the route of administration is very important when evaluating safety, as two studies have shown that intracranial injection of VSV- $\Delta$ M51 can be neurotoxic to Swiss-Webster mice and CD-1 nude mice [48, 61].

## OVERCOMING RESISTANCE OF SOME CANCERS TO VSV

As described above, the oncoselectivity of VSV is largely based on the defective type I IFN-associated antiviral potential of cancer cells compared to normal cells [8, 9]. However, in the last few years, it has become clear that some cancers have intact or even upregulated (compared to normal cells) antiviral signalling, which makes them resistant to VSV and other OVs [9, 62–65]. Several approaches have been developed to overcome resistance of such cancers to VSV.

Our own studies have shown that combining VSV- $\Delta$ M51 with JAK Inhibitor I (pan-JAK inhibitor) [64, 66] or ruxolitinib (JAK1/JAK2 inhibitor) [66] or TPCA-1 (originally described as a selective I $\kappa$ B kinase (IKK)- $\beta$  inhibitor, but we demonstrated that it also directly inhibits JAK1 [66]) dramatically improves VSV- $\Delta$ M51 replication and oncolysis in

all tested VSV-resistant PDAC cell lines [64, 66]. Later, we showed that a distinct subset of 22 IFN-stimulated genes (ISGs) were constitutively expressed in VSV-resistant PDAC cell lines and downregulated by both ruxolitinib and TPCA-1. Further analyses demonstrated that four of these genes (MX1, EPSTI1, XAF1 and GBP1) are constitutively co-expressed in VSV-resistant but not VSV-permissive PDAC cells, thus serving as potential biomarkers to predict OV therapy success. Moreover, shRNA-mediated knock-down of MX1 showed a partial restoration (compared to ruxolitinib treatment) of VSV- $\Delta$ M51 replication in resistant PDAC cells, suggesting that at least some of the identified ISGs contribute to resistance of PDACs to VSV- $\Delta$ M51 [67]. Similarly, several ISGs (IRF-9, IRF-7 and OAS) were found to be constitutively expressed in VSV-resistant human head and neck cancer cells. Combining JAK Inhibitor I or ruxolitinib with VSV or VSV- $\Delta$ M51 enhanced viral infection, spread and progeny yield in a panel of human head and neck cancer cells [68]. Recently, for the first time, an *in vivo* enhancement of oncolytic VSV-GP treatment by ruxolitinib was reported, both in subcutaneous as well as in orthotopic xenograft mouse ovarian cancer models [69]. Importantly, although ruxolitinib inhibits JAK/STAT signalling not only in cancer but also in normal cells, this combination did not cause a significant additional toxicity, compared to VSV-GP treatment alone [69].

In addition to the JAK/STAT pathway, other pathways have also been shown to play a role in VSV resistance. The nuclear factor-kappa B (NF- $\kappa$ B) signalling pathway is involved in immunity, inflammation, cell growth and survival. Constitutive NF- $\kappa$ B activation is common in cancer, and NF- $\kappa$ B has also been shown to enhance type I IFN signalling [70]. Therefore, several studies examined VSV in combination with drugs that inhibit NF- $\kappa$ B signalling, generally IKK inhibitors. IKK inhibitors BMS-345541 and TPCA-1 attenuated the antiviral state in glioma cells against VSV by inhibiting NF- $\kappa$ B activity as well as MX1 and GBP1 (both ISGs) expression, and improved VSV replication and oncolysis [71]. However, inhibition of NF- $\kappa$ B does not enhance OVs in all cancers. Combining VSV with the IKK inhibitor BMS-345541, which previously improved VSV replication in glioma cells, did not do so in myeloma cells [71, 72]. Also, combining VSV with the proteasome inhibitor bortezomib inhibited not only VSV-induced NF- $\kappa$ B activation, but also VSV replication in myeloma cells. Interestingly, *in vivo*, this combination did not inhibit i.t. VSV replication or reduced tumour burden in a myeloma mouse model, emphasizing how effects can be very different *in vitro* and *in vivo* [72]. Our own study of VSV-resistant PDAC cell lines evaluated a panel of 16 inhibitors of different cellular signalling pathways. In addition to JAK inhibitors, Jak Inhibitor 1 and ruxolitinib, we identified the IKK- $\beta$  inhibitor, TPCA-1, as a strong enhancer of VSV- $\Delta$ M51 replication and virus-mediated oncolysis in all tested resistant PDAC cell lines [66]. However, all other tested IKK inhibitors (including BMS-345541) did not improve VSV replication, and our results suggested that TPCA-1 enhances VSV



replication mainly by directly inhibiting JAK1 kinase rather than IKK- $\beta$  [66]. Finally, resistance of some cancer cells to VSV can be broken via activation rather than inhibition of NF- $\kappa$ B signalling. Thus, the histone deacetylase (HDAC) inhibitor Vorinostat (SAHA) treatment in prostate cancer PC3 cells has been shown to stimulate NF- $\kappa$ B signalling, leading to induction of autophagy, suppression of the IFN-mediated response, and subsequent enhancement of VSV replication and apoptosis [73, 74]. Moreover, when NF- $\kappa$ B signalling was inhibited using pharmacological or genetic approaches, VSV replication and cell killing were suppressed [73]. Together, all these studies demonstrate that the role of NF- $\kappa$ B signalling in resistance of cancer cells to VSV is cell type dependent.

VSV has also been combined with natural agents such as herbal supplements curcumin and triptolide. Pre-treatment with curcumin improved VSV-mediated oncolysis of PC3 prostate cancer cells *in vitro* and *in vivo* [75]. This improvement correlated with a decrease in the phosphorylation of NF- $\kappa$ B, increased expression of the anti-apoptotic protein Bcl-xl, and inhibition of the phosphorylation and activation of STAT1, all possibly contributing to an increase in VSV infection [75]. Triptolide treatment increased VSV replication *in vitro* in human prostate cancer cell lines (PC3 and DU145) and human B-lymphoma cell line (Karpas-422), and *in vivo* in a PC3 mouse xenograft model. The study suggested that triptolide inhibited the innate antiviral response by blocking type I IFN signalling downstream of IRF3 activation at the transcriptional level [76]. The exact mechanisms of VSV replication stimulation by curcumin and triptolide are unclear.

Several microtubule-destabilizing agents (colchicine, vinorelbine, nocodazole, albendazole and parabendazole) have also been shown to sensitize 786-0 human renal carcinoma cells to oncolytic VSV [77]. Colchicine increased VSV- $\Delta$ M51 spread and oncolytic activity in resistant syngeneic and transgenic tumour mouse models. Interestingly, the microtubule-destabilizing agents were shown to inhibit translation of type I IFN mRNAs and enhance bystander death by virus-induced cytokines [77].

Several genetic biomarkers of resistance or permissiveness of cancer cells to VSV have been proposed. N-Myc overexpression has been shown to enhance VSV replication and oncolysis in human neuroblastoma cells due to inhibition of ISGs [78]. Oncogenic Ras has been shown to promote VSV-mediated oncolysis due to inhibition of IRF1 [79]. Sequence analysis of mutations in melanoma revealed that BRAF gene mutation status, one of the most frequent driver mutations in melanoma formation, was predictive of enhanced susceptibility to VSV infection [80]. Changes in susceptibility to oncolytic VSV can also occur during progression of prostate cancer. It has been observed that early prostate cancer with PTEN deletion was susceptible to VSV. However, as the cancer progressed, it became more resistant to VSV, and a high level of STAT 1 expression was detected in resistant prostate cancer cells [81]. PTEN has also been shown

recently to play an important role in antiviral immunity [82]. Interestingly, the expression of viral genes in cancer cells as a result of prior infections of patients with other viruses could sometimes improve VSV OV therapy outcome if those viral genes inhibit type I IFN signalling. Previously, infection of cervical carcinoma cancer cells with human papillomavirus (HPV) was shown to improve VSV infection and killing [83]. Expression of HPV-E6 in a VSV-resistant cell line reduced VSV-induced IFN response (IRF7, p-IRF3 and MX2) and significantly improved VSV-mediated cell death [83]. A similar observation was made recently with hepatitis C virus (HCV), as HCC cells, stably expressing HCV core protein (Hep3B-Core), were more susceptible to VSV due to inhibition of type I IFN signalling via HDAC4 downregulation [84].

Although most studies suggest that upregulated antiviral signalling (via type I IFN and/or NF- $\kappa$ B pathways) plays a major role in resistance of some cancers to VSV, it is likely that at least in some cancer cell types other mechanisms could be responsible. Recently, we demonstrated that the most resistant human PDAC cell line in our study, HPAF-II, showed dramatically weaker attachment of VSV independently of type I IFN signalling [85]. Polycation (polybrene or DEAE-dextran) treatment considerably improved attachment of VSV to HPAF-II. Moreover, combining VSV with polycations and ruxolitinib (JAK1/JAK2 inhibitor) successfully broke the resistance of HPAF-II to VSV by concurrently improving VSV attachment and replication [85].

Finally, even if VSV is able to infect and replicate in cancer cells, its oncoselectivity could be compromised if VSV-infected cells are resistant to VSV-mediated cell death. Recently, we showed that VSV- $\Delta$ M51 was able to induce apoptosis efficiently in 7 out of 10 tested human PDAC cell lines, and determined that the VSV-mediated apoptosis activation mechanism depends on both the VSV M protein and the PDAC cell line [86]. Three cell lines constitutively expressing high levels of ISGs were resistant to apoptosis under most experimental conditions, even when VSV replication levels were dramatically increased by Jak inhibitor I treatment [86]. We discuss different approaches to increase direct oncototoxicity of VSV-based OV therapy in more detail in the following section.

## INCREASING DIRECT ONCOTOXICITY

Previously [8], we identified seven major approaches which had been shown to improve the direct oncolytic abilities ('oncototoxicity') of VSV: (i) combining VSV with chemical agents; (ii) VSV encoding tumour suppressor genes; (iii) VSV encoding 'suicide genes'; (iv) VSV recombinants inducing syncytium; (v) combining VSV with radiotherapy; (vi) combining VSV with tumour embolization; and (vii) combining VSV with anti-angiogenic agents. In addition, the increased oncototoxicity can be achieved indirectly via OV-mediated stimulation of antitumour immunity, which will be discussed later.

### Combining VSV with chemical agents

Previously, combining VSV with the chemotherapeutic drug doxorubicin improved therapeutic effect [87]. Our own group combined VSV- $\Delta$ M51-GFP with the chemotherapeutic drug gemcitabine and observed improved antitumour efficacy in a mouse PDAC xenograft model [54]. It has been previously shown that combining VSV with obatoclastax, a B-cell lymphoma 2 (Bcl-2) inhibitor, reversed resistance of cancer cells to VSV-mediated oncolysis [88]. More recently, it was demonstrated that Bcl-2 inhibitors obatoclastax and ABT-737 disrupted Bcl-2/Beclin-1 and Beclin-1/Mcl-1 interactions, which increased induction of autophagy and apoptosis in human chronic lymphocytic leukemia cells and human B-cell non-Hodgkin's lymphoma [89]. Combining VSV $\Delta$ 51-GFP with LCL161, a Smac mimetic compound and inhibitor of apoptosis antagonist, inhibited tumour growth in 76–9 rhabdomyosarcoma syngeneic mice [90]. Some recent studies identified novel targets to improve VSV-mediated oncotoxicity. A novel oncogene was recently found, named cancer upregulated gene 2 (CUG2), which activates Ras and mitogen-activated protein kinases (MAPKs), including ERK, JNK and p38 MAPK [91]. CUG2 was shown to confer resistance to VSV through STAT1-OASL2 signalling [92]. Interestingly, suppressing autophagy genes (Atg5 or Beclin-1) by siRNA increased reactive oxygen species formation and decreased ISG15 expression, which sensitized CUG2-overexpressing A549 human lung cancer cells to VSV-induced apoptosis. Chemical agents targeting autophagy genes such as Atg5 and Beclin-1 would be promising for CUG2-overexpressing cancers [93]. Another novel target is Livin, which is a member of the inhibitors of the apoptosis family. Livin is expressed in a variety of tumours and is hardly detectable in the normal tissue. Knockdown of Livin by siRNA made lung cancer cells more sensitive to VSV treatment [94]. Nrf2 signalling, a protein that regulates the expression of antioxidant proteins has also been shown to increase VSV oncolysis via autophagy-driven suppression of antiviral immunity [95]. Taken together, targeting apoptosis or autophagy genes seems to be a promising approach to improve VSV oncotoxicity.

### VSV encoding tumour suppressor genes

Many OV's have also been combined with p53 tumour suppressor gene therapy [96]. Previously, enhanced oncotoxicity was observed for VSV-M(mut)-mp53, which encodes mouse WT p53 in addition to the mutated M protein [97]. Our own group recently engineered novel VSV recombinants encoding human WT p53 or a chimeric p53-CC, which can evade the dominant-negative activities of endogenously expressed mutant p53 [98]. Interestingly, we showed that VSV-directed TP53 transgene expression dramatically inhibited type I IFN responses in cancer cells and that it occurred through p53-mediated inhibition of the NF- $\kappa$ B pathway. Importantly, VSV-encoded p53 did not inhibit antiviral signalling in normal human pancreatic ductal cells [98].

### Combining VSV with radiotherapy

In a recent study, VSV was combined with radiation therapy (RT). Combining VSV- $\Delta$ M51 with RT and a second round of RT 3 days later significantly reduced tumour growth and increased survival of mice in a head and neck xenograft mouse model compared to either treatment alone. No obvious increase in viral replication was observed [99].

### Combining VSV with tumour embolization

It has been previously shown that tumour embolization (blocking of arterial blood flow in the liver) in combination with i.t. injection of VSV improves therapeutic outcome in multifocal HCC in rats [100]. More recently, systemic perfusion pressure (a key driver of tumour blood flow) was shown to play a crucial role in the successful delivery of systemically administered oncolytic VSV to the tumour cells in myeloma tumour-bearing mice. General anesthesia and exercise were used to decrease and increase mean arterial pressure, respectively. Anesthesia resulted in decreased i.t. infection density, while exercise increased i.t. infection density and uniformity [101].

### Combining VSV with anti-angiogenic agents

Recently, i.t. injection of VSV in combination with anti-angiogenic agent sunitinib (an inhibitor of VEGF-R and PDGF-R) improved therapeutic outcome in prostate, breast and kidney malignant tumours in mice. Sunitinib treatment, in addition to its anti-angiogenic activity, was shown to stimulate VSV replication by suppressing antiviral responses via inhibition of eIF2- $\alpha$  phosphorylation (substrate of PKR) [102]. VSV therapy was also improved in a head and neck xenograft mouse model when combined with vascular disrupting agent ZD6126 [99].

### Other approaches to increase VSV oncotoxicity

Simultaneous infection of cancer cells with VSV and other biological agents can sometimes improve OV therapy outcome if those agents inhibit type I IFN signalling. VSV has been previously combined with the OV vaccinia virus (VV) to improve VSV-mediated oncolysis. This improvement was mainly due to the activity of the VV B18R gene product that antagonizes the innate antiviral response initiated by type I IFNs [103]. Recently, VSV was combined with a non-pathogenic *E. coli* expressing B18R. *In vitro*, VSV- $\Delta$ M51 infection was greatly enhanced by the B18R produced from *E. coli* in HT29 cells. *In vivo*, *E. coli*-B18R increased VSV replication and tumour destruction in HT29 and LLC subcutaneous tumours in athymic mice [104].

Although it is generally expected that enhancing VSV oncotoxicity should benefit OV therapy outcome, this approach can lead to some unforeseen negative consequences. A recent study, in a MPC-11 plasmacytoma model, showed that VSV-based OV therapy can induce tumour lysis syndrome, a condition that arises when large amounts of tumour cells are being killed at the same time and release their contents into the bloodstream. This condition leads to high toxicity (e.g. hyperkalemia, hyperphosphatemia,

hypocalcemia, hyperuricemia, lymphopenia, dehydration, weight loss) and mice died 5–8 days after treatment [105].

## PREVENTING PREMATURE CLEARANCE OF VSV

Circulating nAbs, non-specific host proteins or complement proteins can prematurely neutralize VSV particles. Prior to 2012 [8], we identified three major approaches to address this important issue: (i) physical delivery methods hiding/masking virus from nAbs, other host components or immune cells; (ii) VSVs expressing genes favouring VSV survival; and (iii) combination of VSV with chemicals favouring VSV survival. Several recent papers addressed this important problem.

### Physical delivery methods hiding/masking virus from nAbs, other host components or immune cells

A technology has been developed to hide/mask VSV from Abs called aptamer-facilitated virus protection (AptaVIP) that is based on two types of DNA aptamers: blocking and shielding aptamers. Blocking aptamers bind to antigen-binding fragments of nAbs (anti-nAbs aptamers) and prevent neutralization of a virus; shielding aptamers bind to virions and mask them from recognition by nAbs allowing the virus to attach to and infect cancer cells. This approach increased viral infectivity by more than 70% in the presence of nAbs [106–108]. In another study, covalent modifications of VSV with polyethylene glycol (PEG) or a function-spacer-lipid (FSL)-PEG construct were developed to inhibit serum neutralization of systemically delivered VSV. In mice, PEGylation of VSV provided protection against neutralizing anti-VSV antibodies and improved the persistence of VSV in the blood [109]. VSV stability in the presence of human serum can also be enhanced by shielding VSV with a thin polymer shell synthesized *in situ* onto the viral envelope [110].

### VSVs expressing genes favouring VSV survival

VSV-G can be made serum-resistant through a directed evolution experiment (six viral passages in the presence of human serum) [111]. Another approach is to substitute VSV-G for another glycoprotein that is more resistant to nAbs. The previously discussed VSV-GP, encoding LCMV glycoprotein instead of VSV-G, can escape humoral immunity by not eliciting a nAb response [46]. Maraba virus, in which G protein is about 80% homologous to VSV-G, is relatively resistant to the neutralizing activities of non-immune human serum. The parental VSV and VSV encoding Maraba G (instead of VSV-G) have nearly identical host range properties and replication kinetics. However, in contrast to the parental VSV, the VSV encoding Maraba G was resistant to non-immune human serum [112].

## INDUCING TUMOUR-SPECIFIC IMMUNITY

In the recent years, it became clear that fully effective OV therapy should activate tumour-specific adaptive immune responses, and many new studies focus on improving VSVs'

immunotherapeutic potential [113]. Even though VSV has immunostimulatory abilities by itself, many approaches are being investigated to rationally improve tumour-specific immunity [114]. Most of the studies are aimed at improving tumour-specific T-cell responses, however it is important to mention that studies have shown that the capacity to induce a stronger immune response against a tumour antigen does not always correlate with improved therapeutic efficacy [115].

Recently, a VSV-ΔM51 vector expressing functionally active IFN- $\gamma$  (VSVΔ51-IFN $\gamma$ ) was generated. VSVΔ51-IFN $\gamma$  induced an increase in serum levels of various proinflammatory cytokines (IL-6, TNF- $\alpha$ , MCP-1) and greater activation of dendritic cells. Overall, VSVΔ51-IFN $\gamma$  reduced the number and size of lung tumours in the 4T1 immunocompetent mouse model and demonstrated better efficacy compared to the parental virus. This improved efficacy was lost in immunocompromised animals, suggesting that the mechanism is T-cell-dependent [116].

Previously, VSV-cDNA libraries were used to identify tumour-associated antigens (TAAs) capable of inducing enhanced tumour-specific immunity. The study was done on B16 melanoma tumours and the screen identified three viruses encoding putative TAAs that had a therapeutic effect (neuroblastoma-Ras, cytochrome c and tyrosinase-related protein 1) [117]. In a more recent study by the same group, different VSV-TAAs consisting of VSV expressing hypoxia-inducible factor [HIF]-2 $\alpha$ , Sox-10, c-Myc, and tyrosinase-related protein 1 showed a good therapeutic effect against B16 intracranial tumours [118]. The VSV-TAA approach has also been combined with stereotactic ablative radiation therapy (SABR). SABR can control or cure local clinically detectable and accessible tumours through direct cell ablation, whereas VSV-TAA can generate T-cell responses that can clear subclinical metastatic tumours [119]. The combination of VSV-TAA engineered to express the endogenous melanocyte antigen glycoprotein 100 (gp100) with adoptive T-cell transfer (transfer of T-cells into a patient) reduced melanoma burden and induced antitumour immunity [120]. The VSV-TAA (VSV-ova) approach was also combined with the TLR-4 agonist lipopolysaccharide to activate different innate immune pathways [121]. This combination therapy improved both general and specific T-cell activation, however it also led to rapid toxicity due to rapidly elevated serum levels of tumour necrosis factor- $\alpha$  and interleukin 6. These results emphasize how some combination therapies can cause unexpected dangers [121].

PD-L1 is upregulated in many cancers and inhibits cytotoxic T-cell activity by binding to the programmed death 1 (PD-1) receptor on T-cells. Many immune-checkpoint inhibitors have been developed in the past years that target PDL-1 or PD-1 [122]. Some studies have shown that inhibiting PD-1 or PDL-1 can improve VSV therapy. Combining VSV-TAA with the checkpoint inhibitor anti-PD-1 antibody enhanced treatment by improving Th1 response [118]. Moreover, double checkpoint inhibition therapy

(anti-PD1 and anti-cytotoxic T lymphocyte antigen 4 (CTLA4)) enhanced treatment with VSV-TAA even further by improving Th17 response [118]. In another study, combining VSV-mIFN $\beta$ -NIS with anti-PD-L1 antibody enhanced antitumour activity by increasing tumour-infiltrating CD4 and CD8 cells [123]. Another group also saw a robust tumour growth inhibition when VSV-IFN $\beta$  and checkpoint inhibitors were combined [124]. Combining VSV-TAA expressing a cDNA library of melanoma antigens (VSV-ASMEL) with reovirus and anti-PD-1 treatment did improve survival in mice [125]. In these studies mice were first treated with VSV (one or multiple injections over several days) and a few days later treated with checkpoint inhibitors (multiple injections over several days). In another study, VSV-TAA and adoptive cell therapy were combined with checkpoint inhibitors, however it did not improve survival of mice stressing that checkpoint inhibitors do not always improve VSV therapy [126].

## MARABA VIRUS

While this review mainly focuses on VSV, there is another vesiculovirus closely related to VSV, Maraba virus, which is also being developed into a promising OV. The most commonly used Maraba virus recombinant used is the engineered attenuated Maraba strain MG1, containing both G protein (Q242R) and M protein (L123W) mutations. MG1 retains its killing potency in cancer cells yet is attenuated in normal cells *in vivo* [127]. MG1 has also been shown to boost natural killer cells activity via direct infection and maturation of dendritic cells, which led to reduction of B16lacZ lung metastases in C57Bl/6 mice [128].

MG1 showed very good efficacy in various *in vitro* and *in vivo* cancer models, including colon, ovarian and sarcoma tumour models [127, 129, 130]. As with VSV, certain approaches have been developed to improve Maraba virus therapy. MG1 was combined with commonly used chemotherapeutic agent paclitaxel, and this combination approach resulted in an increase in virus production and killing *in vitro* and *in vivo* in a mouse breast cancer model [131]. As mentioned above, LCMV glycoprotein is known for its inability to induce production of early neutralizing antibodies in the infected hosts. To improve Maraba virus-based OV, the LCMV-encoding Maraba virus (MRB LCMV GP) was generated and tested in a mouse colon cancer model [132]. It was observed that MRB LCMV GP was neutralized by anti-LCMV-GP antibodies in a complement-dependent manner. However, complement inhibition *in vivo* increased the effective dose of MRB LCMV GP that was delivered to tumours [133]. MG1 has also been engineered to express IL12 (MG1-IL12), and this recombinant virus reduced tumour burden and improved survival in a mouse colon cancer model of peritoneal carcinomatosis due to enhanced NK activity [132]. MG1 was also engineered to express a melanoma-associated tumour antigen. While unable to prime detectable responses against a melanoma-associated tumour antigen, this engineered MG1 displayed a potent ability to boost pre-existing tumour-specific CD4<sup>+</sup> and

CD8<sup>+</sup> T-cell immunity and improved survival in melanoma lung and brain tumour-bearing animals [134]. Maraba virus is currently being tested in Canada in phase I/II clinical trials (ClinicalTrials.gov trials NCT02285816 and NCT02879760).

## CONCLUDING REMARKS/FUTURE DIRECTIONS

VSV continues to be a very promising OV, but many important questions remain to be answered. First of all, it is not fully understood what cancers are the best targets for VSV-based OV therapy. In the last 5 years VSV has been tested in many novel cancer models, and VSV showed promising results in the following cancer models: endometrial cancer [135], metastatic colon cancer [136], neuroendocrine tumours [137], small cell ovarian carcinoma [138], metastatic Erwing sarcoma [139], malignant ascites [140], metastatic lesions associated with advanced prostate cancer and HCC with hepatic fibrosis [141], murine plasmacytoma [105]. Our laboratory tested VSV in an immunocompetent mouse model of PDAC overexpressing or not expressing human mucin 1 (MUC1), a major marker for poor PDAC (and some other cancers) prognosis in patients [142]. *In vivo* administration of VSV- $\Delta$ M51-GFP resulted in significant, but transient, reduction of tumour growth for tested mouse PDAC xenografts (+MUC1 or MUC1 null) [54]. A recent review describes in detail murine tumour models for VSV efficacy studies [143].

Understanding how VSV spreads in the tumour and host is also important. Since 2012, many novel techniques have been developed to track VSV more efficiently and easily. Vectors equipped with HSV-1 thymidine kinase reporter are detectable by positron emission tomography after application of an appropriate radionuclide-labelled tracer [144]. Another commonly used reporter is the thyroidal NIS. A system using very high-resolution non-invasive *in vivo* micro single-photon emitted computed tomography/computed tomography (microSPECT/CT) imaging was developed to determine the i.t. distribution of NIS in tumours infected with VSV-mIFN $\beta$ -NIS and addition of the radionuclide <sup>99m</sup>Tc-labelled tracer [145].

It is still unclear what the most efficient delivery methods for VSV into tumours are. Recently, VSV that can bind to myeloid-derived suppressor cells (MDSCs) was engineered to use them as a delivery vehicle. MDSCs are superior to other immune cell types in preferential migration to tumours in comparison to other tissues. Improving VSV binding efficiency to MDSCs extended the long-term survival of mice bearing metastatic colon tumours compared to systemic administration of WT VSV alone [146].

All these questions are very complex and more studies are needed. At least some studies could benefit from mathematical models that already make important contributions to VSV-based OV therapies [147–149].

Another important question is the possible impact of VSV-based recombinant OV's on the environment if such recombinant VSVs escape into nature. Recently, the pathogenicity and transmissibility of oncolytic VSV-IFN $\beta$ -NIS was studied using a swine model, as VSV is considered an animal pathogen and could be of concern for livestock. VSV-IFN $\beta$ -NIS was both non-pathogenic and not transmissible in pigs, a natural host [150]. This study and many others support further clinical development of oncolytic VSV as a safe therapeutic for human cancer, while the results from the multiple VSV and Maraba virus clinical trials are eagerly awaited.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

#### Ethical statement

Ethics approval was not obtained because this is a systematic review of the literature.

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