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# Oncolytic viruses: From bench to bedside with a focus on safety

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**Abbreviations:** CAR, Coxsackie Adenovirus receptor; CD, cytosine deaminase; CEA, carcinoembryonic antigen; crHAdV, conditionally replicating HAdV; CVA, Coxsackievirus type A; DAF, decay accelerating factor; DNA, DNA; dsDNA, double stranded DNA; dsRNA, double stranded RNA; EEV, extracellular enveloped virus; EGF, epidermal growth factor; EGF-R, EGF receptor; EMA, European Medicines Agency; FDA, Food and Drug Administration; GBM, glioblastoma multiforme; GM-CSF, granulocyte-macrophage colony-stimulating factor; HA, hemagglutinin; HAdV, Human (mast)adenovirus; HER2, human epidermal growth factor receptor 2; hIFNβ, human IFN β; HSV, herpes simplex virus; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; IRES, internal ribosome entry site; Kb, kilobase pairs; KRAS, Kirsten rat sarcoma viral oncogene homolog; MeV, Measles virus; mORV, Mammalian orthoreovirus; mORV-T3D, mORV type 3 Dearing; MuLV, Murine leukemia virus; NDV, Newcastle disease virus; NIS, sodium/iodide symporter; NSCLC, non-small cell lung carcinoma; oHSV, oncolytic HSV; OV, oncolytic virus; PEG, polyethylene glycol; PKR, protein kinase R; PV, Polio virus; Rb, retinoblastoma; RCR, replication competent retrovirus; RCT, randomized controlled trial; rdHAdV, replication-deficient HAdV; RGD, arginylglycylaspartic acid (Arg-Gly-Asp); RNA, ribonucleic acid; ssRNA, single stranded RNA; SVV, Seneca Valley virus; TGFα, transforming growth factor α; tk, thymidine kinase; VGF, Vaccinia growth factor; VSV, Vesicular stomatitis virus; VV, Vaccinia virus

Oncolytic viruses are a relatively new class of anti-cancer immunotherapy agents. Several viruses have undergone evaluation in clinical trials in the last decades, and the first agent is about to be approved to be used as a novel cancer therapy modality. In the current review, an overview is presented on recent (pre)clinical developments in the field of oncolytic viruses that have previously been or currently are being evaluated in clinical trials. Special attention is given to possible safety issues like toxicity, environmental shedding, mutation and reversion to wildtype virus.

## Introduction

Oncolytic viruses (OVs), reported first halfway the previous century, have undergone a tremendous evolution from anecdotal experimental and clinical efficacy to state-of-the-art clinical trials employing recombinant viruses in the last decade. With the advent of reverse genetics techniques, modifications attributing to efficacy and safety have marked the introduction of new generations of recombinant OVs. Most recent developments have focused on conditional replication in tumor cells, expression of (therapeutic) transgenes as well as targeting and/or delivery of OVs.

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In this review, an overview is given of the current state of affairs concerning OVs that are being developed toward clinical trials or that are used in clinical trials. Besides information on (pre)clinical efficacy, special focus is given to the safety of these new agents, specifically toxicity, environmental shedding and mutation rates or reversion to wild type virus. A summarizing overview is presented in **Table 1**.

Safety of OVs primarily relates to the toxicity of the administration, especially when thinking about high dose intravenous application. In addition, environmental shedding of infectious viruses is also of concern, not only for perceived safety, but also for regulatory purposes. To this end, in general, OVs should be generated which preferably are tumor-specific and have low to no shedding upon (systemic) administration.

## Family Herpesviridae: Herpes Simplex Virus 1 (HSV)

HSV-1 causes herpes labialis (cold sores) in humans. HSV-1 was one of the first viruses to be developed into a recombinant oncolytic virotherapeutic vector. The large HSV genome is easy to manipulate and allows insertions of multiple additional transgenes. Furthermore, HSV infects and replicates in most tumor cell types and spreads throughout the tumor. If needed, viral replication can be hampered with anti-HSV medication (Acyclovir). Because HSV is neurotropic and causes a latent infection, most genetic modifications of oncolytic HSV (oHSV) have first focused on this potential safety issue.

To increase safety, tumor-specific oHSVs have been generated using 3 main strategies, as reviewed earlier by Campadelli-Fiume et al.[1] These strategies include first attenuation by means of

**Table 1.** Characteristics of oncolytic virus platforms

| Virus | Packaging capacity | Oncoselectivity | Optimized | | Prime examples | Status clinical trials | Tox | Shed | Mutation | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | with Arming | with Targeting | | | | | | |
| HSV | large | dividing cells (UL39) PKR response (γ1–34.5) downregulation MHC-I (α47) | yes | yes | talimogene laherparepvec rQNestin34.5 NV1020[1], G207 | pending FDA/EMA | low | limited | seldom | 1–18 |
| HAdV | large | E1B-55kDa late viral RNA export Rb pathway defects overactive KRAS pathway | yes | yes | ONYX-015, Gendicine Oncorine, Delta24 ColoAd1, Telomelysin | phase III | low | yes | no | 20–26,28–35,40–45,48–50, 55,56,58–67 |
| MeV | 1–2 (large) transgenes | CD46 receptor innate immunity defects | yes | yes | MeV-CEA, MeV-NIS | phase II | low | no | no | 69–74 |
| NDV | 1–2 transgenes | innate immunity defects apoptosis dysregulation | yes | yes | MTH68, 73-T, Ulster PV701, HUJ, rNDV $F_{3aa}$ | phase II | low | yes | no | 75,77–87 |
| VSV | 1–2 transgenes | type I IFN response defects abnormal translation machinery | yes | yes | VSV-hIFNβ | phase I | low | no | yes | 88,90–100 |
| CVA | N/A* | ICAM-1/DAF receptor overexpression | N/A* | N/A* | CAVATAK | phase II | low | no info | yes | 103–105 |
| PV | N/A | CD155 receptor overexpression | no | no | PVS-RIPO | phase I | low | no | yes | 106–113 |
| SVV | 1 transgene | Unknown, neuroendocrine tumors | no | no | recombinant SVV-001 | phase II | low | no | yes | 114–120 |
| VV | 1–3 transgenes | overactive EGF-R pathway cellular tk expression type I IFN response defects | yes | no | Pexa-vec, vvDD, GL-ONC1 | phase II | low | yes | no | 123–125,127–134 |
| mORV | N/A | overactive KRAS pathway | yes | no | mORV-T3D REOLYSIN | phase II | low | yes | yes | 135,137–139,142–144 |
| MuLV | 1 transgene | dividing cells | yes | no | Toca 511 | phase II | low | no | no | 145–150 |

Overview of different oncolytic virus platforms and their characteristics. Prime examples include specific viruses that have been evaluated extensively in clinical trials or have promising potential based on preclinical experiments. Mutation describes the possibility of viruses to revert to wildtype, to recombine with wildtype virus, or mutate to more virulent (quasi)species. Abbreviations: HSV, Herpes simplex virus; HAdV, Human mastadenovirus; MeV, Measles virus; NDV, Newcastle disease virus; VSV, vesicular stomatitis virus; CVA, Coxsackievirus A; PV, Poliovirus; SVV, Seneca Valley virus; VV, Vaccinia virus; mORV, Mammalian orthoreovirus; MuLV, Murine leukemia virus; N/A, non-applicable; tox, toxicity; shed, shedding.
*= CVA does not have a reverse genetics system.

conditional replication in tumor cells through deletion of viral genes that are essential for viral replication in non-dividing cells (UL39), counteract the PKR response ($\gamma_1$-34.5) or contribute to immune evasion ($\alpha$47). Secondly, to increase the oncolytic efficacy (and often co-incidentally improve safety), oHSVs have been armed with immune stimulatory genes to boost local cytotoxic immune responses or other therapy enhancing transgenes. Thirdly, targeting by tropism or transcription specificity has been applied to limit virus infection even further to only tumor cells.

First generation oHSVs harboring the aforementioned genetic deletions have shown to be safe regarding toxicity based on their attenuation in normal cells. However, they are also attenuated in tumor cells and thus less cytotoxic. Recent strategies have focused on improving the targeting of less attenuated oHSVs by changing tropism or transcription specificity. Glycoprotein D is the receptor binding protein of HSV-1, and fusion of this glycoprotein with a heterologous ligand can retarget the virus to the tumor-specific receptor of choice. This tumor specific targeting is enhanced by detargeting the normal receptor.[2,3] Examples include IL−13R$\alpha$2, HER2, and EGF-R.[4-6] Using transcriptional targeting, tumor specificity has been enhanced by placing viral genes under the control of tumor-specific promoters. A promising example is rQNestin34.5, which has the expression of the $\gamma_1$-34.5 gene under the control of the glioma-specific nestin promoter, which restores viral replication and cytotoxicity only in glioma tumors.[7]

Several early generation oHSVs (talimogene laherparepvec, HSV1716, NV1020, G207, M032, rRp450 and others) have already been evaluated in clinical trials.[1] Most of these trials have demonstrated a good safety profile and treatment benefits were also observed. Talimogene laherparepvec (oHSV with deletions in $\gamma_1$-34.5 and $\alpha$47, armed with GM-CSF) has recently undergone evaluation in a phase III clinical trial in patients with advanced or metastatic melanoma. Probably this will be the first oncolytic virus to obtain FDA approval, while a marketing authorization application for the European Union has just been submitted to the EMA.[8]

Preclinical evaluation employing intra-organ (brain or prostate) injection with oHSV in non-human primates demonstrated no shedding of virus, which points to limitation of oHSV replication to injection sites.[9,10] This was confirmed by early clinical trials in patients injected intratumorally (glioma) with oHSVs: sporadic shedding of HSV in saliva was noted, but this was shown to be wildtype virus as opposed to the injected oHSV.[11,12] Other studies have observed limited leakage of oHSV from injection sites up to 2 weeks post treatment, without other excreta containing viable oHSV.[13-15] Intra-arterial hepatic injection did not result in detectable environmental shedding either.[16] Thus oHSV fulfills the criteria for safety with regards to shedding. A possible concern is that an oHSV recombines with a wildtype endogenous virus. If the oHSV carries heterologous genes, the recombinant would have to arise by illegitimate recombination – an extremely rare event that cannot be replicated *in vitro*.[17] Spontaneous reversion of oHSVs with deleted viral genes to wildtype virus is not possible. However, compensatory mutations can arise, which can compromise safety, but so far resulting HSV mutants have been highly attenuated.[18] These compensatory mutations can be of concern when evaluating the safety of newer generation oHSVs, because more virulent oHSVs could arise.

## Family Adenoviridae: Human Mastadenovirus (HAdV)

HAdVs can be associated with different diseases in humans: (upper) respiratory tract infection (mainly species B and C), conjunctivitis (species B and D) and gastroenteritis (species F and G). Sporadically, HAdVs can cause viral meningitis, encephalitis or hemorrhagic cystitis.[19]

Because of its association with mild disease and a relatively easy to manipulate genome (as compared to other HAdV types/species), most work on HAdV as vector for (cancer) gene therapy has been done with serotype 5 of species C. HAdVs have distinct advantages as a gene transfer vector, including high transfection efficiency of cells irrespective of their growth status. The genome of HAdVs is easy to manipulate for retargeting and insertion of transgenes, and efficient production of high titer virus stocks is possible. Disadvantages include high immunogenicity of prevalent serotypes with pre-existing immunity, and transient expression of the transgene due to dilution of replication-deficient HAdV (rdHAdV) episomes upon cellular division.[20]

In case of oncolytic HAdV vectors, replication of the virus is thought to be advantageous because of direct cancer cell killing induced by viral replication, and due to which the number of administrations needed for effective treatment can be reduced. Efforts to improve safety have been made in developing conditionally replicating HAdVs (crHAdVs), with specific and higher replication in cancer cells. Early examples are ONYX-015 (*dl1520*) and H101, which have a deletion of E1B-55kDa (and a deletion in E3 for H101), normally responsible for p53 binding and inactivation.[21] The tumor specific replication of ONYX-015 was later shown to be due to loss of E1B-55kDa-mediated late viral RNA export, rather than p53-inactivation.[22] In a similar approach, newer crHAdVs have been created exploiting the defects in Rb pathways in cancer cells by deleting the Rb-binding E1A-CR2 region, creating *dl922–947*, also known as Delta24.[23] Additional modifications in Delta24 have been created and successfully evaluated for oncolytic efficacy,[24,25] as well as oncolytic crHAdVs which target cells with an (hyper)active KRAS pathway,[26] or with YB-1 overexpression,[27] limiting crHAdV replication to cancer cells. A different approach for creating crHAdVs is using cancer- or tissue-specific promoters to limit expression of essential early HAdV genes to specific celltypes and/or tissues.[28,29]

Like other oncolytic viruses that have undergone extensive development, crHAdVs have also been armed with transgenes, often under the control of a tissue/cancer-specific promoter. Examples include i.e. immunomodulatory, pro-apoptotic or pro-drug converting enzyme genes.[30-33]

Despite their capacity to achieve tumor infection in animal models and in clinical trials, the therapeutic efficacy of rdHAdVs

in clinical trials has been disappointing; Advexin and Cerepro have not been approved by the FDA and EMA, although a similar agent called Gendicine has been approved for cancer therapy in China.[34,35] The discrepancy between preclinical and clinical studies using HAdV-5 could be explained by the differences in expression of CAR in primary tumors compared to established laboratory cell lines.[36] In addition, off-target toxicity by sequestration in mainly the liver is a serious concern, even when HAdVs are blinded for CAR, because this can lead to serious liver damage.[37-39] Hexon mutations or even complete exchange of hexons have been shown to reduce liver sequestration and transduction dramatically.[40,41] Other strategies used to circumvent liver sequestration include PEGylation or polymer/dendrimer coating of HAdV virions, and cell-based or magnetic/liposomal nanoparticle delivery. To circumvent the limitation of low CAR expression in (tumor) cells, retargeting has also been applied to HAdVs, permitting CAR-independent infection.[42] The retargeting strategy can also circumvent existing humoral immunity for HAdV-5 in the general population, and contributes to the prevention of liver sequestration as described above. Other examples include conjugation with anti-knob or anti-penton/hexon antibodies or adapters with retargeting ligands, pseudotyping or xenotyping with (chimeric) fiber knobs or capsids, peptide presentation (RGD or other), Affibody targeting, knob-less HAdVs and genetically modified capsids and/or fiber knobs.[20] More recently, efforts have also been made to develop HAdVs fully based on other serotypes, most notably HAdV-3, or even non-human AdVs.[43-45] Using 'directed evolution' or 'accelerated evolution' strategies, ColoAd1 and other crHAdVs have been generated which appear to be more potent than their parental HAdV-5 based vectors.[46,47]

A total of 458 clinical trials employing HAdV-mediated gene therapy have been reported to date. ONYX-015, H101 (Oncorine) and other first-generation oncolytic crHAdVs have gone through several phase I/II trials without relevant signs of high grade toxicity but also without significant therapeutic effects, resulting in discontinuation of further trails.[48] More recent clinical trials employing new generations of crHAdVs like RGD retargeted oncolytic crHAdVs,[20,49,50] crHAdV-5/3 chimeric vectors,[32,51-54] ColoAd1,[55] hTERT-promoter driven crHAdV-5 vector Telomelysin,[56] E2F-1-promoter driven CG0070[33], Rb-targeted crHAdV expressing hyaluronidase (VCN-01)[57] and crHAdV vectors expressing immunomodulating genes have shown safety (low toxicity) with some promising preliminary results.

In general, the use of early generation oncolytic crHAdVs appears to be reasonably safe with low toxicity when administered locally and at lower doses systemically. However, the development of newer generations of crHAdVs expressing transgenes, having altered capsids or different promoters can dramatically alter this perceived safety. Shedding of crHAdVs from injection sites and patient excretions, although not always reported, has been observed in several (pre) clinical trials, and increases with higher doses and systemic administration.[49,58-64] Shedding of HAdV vectors could result in homologous recombination between AdVs of the same subgroup, which occurs with high efficiency during growth in co-infected cultured cells, and there is evidence of recombination events in humans as well.[65-67] Theoretically, homologous recombination between wildtype AdVs and recombinant crHAdVs could lead to new wildtype AdVs that e.g. possess transgenes, or worse, have expanded tissue tropism due to retargeting strategies. However, to date such recombination has never been detected in any clinical trial.

## Family Paramyxoviridae: Measles Virus (MeV)

MeV is highly contagious via the respiratory route and is responsible for high morbidity and mortality rates in immune naive subjects.[68] Large-scale vaccination programs with live-attenuated MeV have been very successful. An extensive safety record has been established for the use of vaccine strains of MeV in humans.[69]

Most (pre) clinical research with oncolytic MeVs have used the attenuated vaccine Edmonston strain, which is perceived to be very safe in terms of toxicity.[70] The cancer selectivity of MeV stems from overexpression of the MeV receptor CD46 on malignant cells.[71] Recombinant MeV can accommodate and maintain large sizes of foreign genetic material with good genetic stability *in vitro* and *in vivo*, and MeVs expressing transgene(s) have shown good genetic stability upon passaging. Both arming and targeting strategies have been used to improve efficacy of MeV in a wide array of malignancies.[70]

Completed and ongoing clinical trials in patients with T cell lymphoma, ovarian cancer or glioblastoma multiforme have first used wild type MeV and later recombinant MeV expressing marker genes CEA and NIS.[72-74] Intratumoral, intraperitoneal and intravenous administration have been reported using doses up to $10^9$ infectious viral particles without dose limiting toxicity or MeV induced immunosuppression.[72-74] Although wildtype MeV can cause potentially serious disease, attenuated MeV vaccine strains like Edmonston have an excellent safety record.[69] In clinical trials with rMeV-CEA, no evidence was seen of shedding in sputum and urine samples of patients who were intraperitoneally injected.[73] Spread of oncolytic MeV in the general population is highly unlikely since most individuals in industrialized countries are immunized, although herd immunity is currently waning with declining vaccination percentages. As noted, the oncolytic MeV of choice to date has been of the Edmonston strain, which has a good safety profile without capability of causing overt disease.

## Family Paramyxoviridae: Newcastle Disease Virus (NDV)

NDV is an avian virus, and as such causes no serious disease in humans.[75] NDV strains are categorized in 3 different groups based on the severity of the disease they cause in birds: lentogenic, mesogenic and velogenic.[76] NDV has been shown to be very safe with regards to toxicity in tumor models using mice or rats, even when used in high dose and injected intravenously, and

NDV also appears to be safe for high dose administration in humans, with no serious adverse events noted in early clinical trials.[77] Several wildtype NDV strains have shown (limited) antitumor activity without major side effects in phase I–II clinical trials for patients with various types of solid cancer.[77]

Using recombinant NDVs, the oncolytic efficacy has been improved by increasing the virulence of the virus and the expression of immunomodulating or apoptotic transgenes. In addition, tumor cells are targeted with modified attachment proteins and combinations with other treatment modalities, most recently immune checkpoint blockade.[78-85] Clinical trials with these improved viruses have not yet been described, but pre-clinical data indicate efficacy with low toxicity in multiple tumor models for several solid malignancies, including pancreatic adenocarcinoma.[78-86]

Virulent NDV strains pose an environmental risk, as birds (specifically poultry) are very susceptible to infection with mesogenic or velogenic strains. A preclinical study evaluating lentogenic and mesogenic oncolytic NDV injected intravenously in non-human primates showed i.v. administration of the virus to be safe without relevant toxicity of high dosages, although relevant shedding was noted.[87]

## Family Rhabdoviridae: Vesicular Stomatitis Virus (VSV)

VSV is the causative agent of vesicular stomatitis in cattle, causing a mild fever and the formation of blister−like lesions on the inside of the mouth, the lips, nose, hooves and udder.[88]

Compared with other oncolytic viruses, VSV has some distinct advantages: first of all a well-studied biology with relative replication independency of cell cycle and a specific receptor. Secondly, VSV produces high virus yields in a wide range of cell types, it replicates intracytoplasmatic without risk of genomic integration, it harbors a small and easy to manipulate genome, and there is no pre-existing immunity in humans.[88] VSV infection in humans is generally asymptomatic and limited to people having direct contact with VSV.[88] A single case of VSV strain Indiana related encephalitis in humans has been reported.[89] VSV oncoselectivity is based largely on defective or reduced type I IFN responses in tumor cells,[90] although abnormal translation machinery and other cellular proteins have also been shown to play a role.[91,92] All 3 strategies previously described (conditional replication, arming, and targeting) have been employed to increase efficacy of VSV.[88] Furthermore, combination therapy has been described with different other therapies. Finally, optimizing delivery and distribution of oncolytic VSVs has been evaluated using cell-based carriers and aptamer or PEGylation of virions. Hastie & Grdzelishvili published an excellent overview of abovementioned strategies and resulting oncolytic VSVs in 2012.[88]

A recent study in purpose-bred beagle dogs showed that a dose up to $10^{10}$ $TCID_{50}$ of VSV-hIFNβ was well tolerated, with mild adverse events with the exception of one dog that received $10^{11}$ $TCID_{50}$ which developed severe hepatotoxicity and shock leading to euthanasia.[93] A following study testing VSV-hIFNβ on rhesus macaques via intrahepatic injection did not result in neurological symptoms and is considered to be safe enough to proceed into phase I clinical trials, which are currently ongoing in humans and pet dogs.[94,95] With regards to shedding, no VSV RNA was detected in buccal swabs taken from non-human primates after intrahepatic injection with VSV-hIFNβ.[94] Theoretically, VSV mutants harboring mutations in their M or G gene (making them oncoselective and abolishing neurotropism) could revert to wildtype VSV upon passaging. Also, VSVs expressing attenuating transgenes like hIFNβ can acquire mutations in this transgene, which has been shown in several studies.[96-98] Furthermore, oncolytic VSVs have been shown to optimize targeting to glycoproteins upon passaging in tumor cells,[99] and to mutate expressed transgenes to optimize replication.[100] These examples have strangely not been perceived as a safety problem, but should be taken into account in future (pre)clinical trials.

## Family Picornaviridae: Coxsackievirus (CVA)

Coxsackieviruses can be divided into 2 groups (A and B) based on their pathogenicity in mice. The best known example of CVA-related human disease is hand, foot and mouth disease, caused by CVA-16.[101] CVA-21 causes upper respiratory tract infections in humans, and it is considered one of the 'common cold' viruses.[102] Similarly to rhinoviruses, CVA-21 binds to ICAM-1 and additionally needs DAF-attachment for productive viral infection.[103] Given that ICAM-1 and DAF are overexpressed in melanoma cells, efforts to evaluate the oncolytic potential of wildtype CVA-21 (and other coxsackieviruses) have mainly focused on this disease.[104]

Currently ongoing phase I/II clinical trials employing intratumoral injection of wildtype CVA-21 (CAVATAK) in Australian patients with advanced melanoma are showing promising preliminary results.[105] All (pre) clinical work so far has been conducted with wildtype CVA, while no progress has been made regarding conditional replication, arming or targeting.

Clinical trials thus far have not led to serious adverse events. No information is available regarding shedding. When considering non- or low-pathogenic coxsackieviruses for oncolytic virotherapy, environmental risks are considered to be low. However, when using viruses that do cause (severe) disease in humans, care should be taken to evaluate and/or attenuate these new vectors.

## Family Picornaviridae: Poliovirus (PV)

The vast majority of PV infections remain asymptomatic in humans, but in 1–2% of cases infection results in neurological complications. Clinical polio syndrome is dominated by flaccid paralysis, due to cell tropism of PV for lower motor neurons in the spinal cord and brainstem expressing $CD155/Necl^{-}5$.[106] Overexpression of CD155 has also been shown in (neuro) ectodermal tumors, and transcriptional upregulation has been linked to signaling pathways commonly affected in malignancy,

including Raf-Erk-Mnk signaling.[107,108] The neuropathogenicity of PV is dependent on the neuronal cell-type-specific function of its IRES element, which assures initiation of translation in a 5' end- and cap-independent manner. Mutations in the IRES genomic region or exchange with other viral IRES counterparts result in markedly neuro-attenuation in CD155-transgenic mice and non-human primates, without reducing the cytopathogenicity in malignant cell types that express CD155.[109]

Most preclinical research has been performed with PVS-RIPO, a recombinant PV type 1 (Sabin vaccine) strain with the IRES element of human rhinovirus type 2. PVS-RIPO has shown oncolytic efficacy in immune-deficient xenograft rat and mouse models of malignant glioma.[110]

Currently a phase I clinical trial is ongoing with intratumoral infusion of PVS-RIPO in patients with recurrent GBM showing durable responses.[111] Extensive evaluation in non-human primates has shown PVS-RIPO to be safe for intraspinal and intrathalamic injection, without observations of extraneural replication or shedding.[109,112] No serious adverse events have been observed so far in an ongoing phase I clinical trial.[111] One of the biggest concerns with PV is the inherent genomic instability of picornaviruses and thus the possible reversion to wildtype pathogenicity. PVS-RIPO has been evaluated extensively for genomic instability by e.g., serial passaging *in vitro* and *in vivo* and it was shown that escape mutants reverting to neuropathogenic virulence in the CD155-transgenic mouse model do arise.[113] Similar mutants have not been observed in other animal models, which makes it unclear what the importance of this preclinical finding is in relation to currently ongoing clinical trials in humans.

## Family Picornaviridae: Seneca Valley Virus (SVV)

SVV was first isolated at Genetic Therapy Inc. (Gaithersburg, MD) as a contaminant from cell culture media and is presumed to be introduced via bovine serum or porcine trypsin source.[114,115] Serum samples taken from different farm animal populations indicated that (healthy) pigs and other animals are exposed to SVV. However, attempts to infect pigs with SVV isolates failed to demonstrate any specific disease. SVV does not infect humans but does propagate in tumor cells with neuroendocrine features, giving the virus a safe profile for use in virotherapy.[114,115] Since its introduction as an oncolytic virus in 2007, SVV has shown preclinical efficacy in nude mice xenograft models for several malignancies.[114-118]

In a phase I clinical trial employing an intravenous dose escalation in patients with neuroendocrine tumors, SVV had (marginal) treatment benefits without causing serious adverse events when administered even in high dose ($10^{11}$ viral particles/kg).[119] A phase II RCT in patients with extensive stage NSCLC and a phase I dose escalation trial in pediatric patients with neuroblastoma, rhabdomyosarcoma or rare tumors with neuroendocrine features are currently underway.[120]

Recent reports indicated that, although the natural host is still uncertain, this virus seems safe with regards to toxicity for use as oncolytic virotherapy in (pediatric) patients.[119] Analysis of samples obtained from researchers in close contact with phase I clinical trial patients revealed the absence of neutralizing antibody titers, which implicates that imposed hygiene policies were effective.[119] However, detailed evaluation of shedding was not performed, and should be determined in future clinical trials.

## Family Poxviridae: Vaccinia Virus (VV)

VV infection induces a strong cytotoxic T lymphocyte response and neutralizing antibodies without causing significant disease in humans.[121] As an oncolytic virus, VV has the advantage of fast replication and cell lysis with a broad cell/tumor tropism. Furthermore, it lacks genomic integration, and shields extracellular enveloped VV virions from host immunity resulting in capability of (systemic) spreading between tumors. Lastly, it also harbors a large genome packaging accommodation.[122]

Several strategies have been described to target oncolytic VV specifically to tumor cells. The VV protein VGF is homologous to cellular growth factor EGF and transforming growth factor α (TGFα) and can stimulate the cell for enhanced viral replication through EGF-R. Deletion of the VGF gene will result in a VV that is targeted to cells with inherent EGF-R pathway activity, which is often observed in cancer cells.[123] J2R gene (encoding for viral tk) deletion similarly results in a VV that is dependent on overexpression of cellular tk, which is also often observed in cancer cells.[124] The combination of VGF and tk gene deletion is known as vvDD and results in an even more selective oncolytic VV, adding to the safety profile.[123] VV gene B18R binds to the IFN receptor and can thereby inhibit the cellular antiviral innate immune response. Deletion of B18R thus leads to selectivity for IFN-deficient cells.[125] A56R gene encodes for HA and deletion results in severe (neuro)-attenuation.[126]

Arming of VV has also been described, e.g. with immune stimulators, apoptotic proteins, anti-angiogenic antibodies/proteins, ECM proteases and prodrug-converting enzymes.

Early clinical trials employing non-recombinant vaccine strains of VV have shown safety when injected superficially into melanoma tumors, while local control of bladder cancer was also noted.[127,128] JX-594 (tk gene deleted, GM-CSF expressing VV Wyeth; Pexa-Vec)[129] has been evaluated in phase I-II clinical trials for patients with metastatic melanoma, (primary) liver tumors, lung, colorectal and various other solid cancer types. GLV-1h68 (GL-ONC1) is currently being investigated in several phase I clinical trials.[130,131]

Clinical trials with oncolytic VV have thus far reported good safety with regards to toxicity with minor side effects like transient low-grade fever and local pain. Commonly, live vaccinia virus is shed from skin injection sites after vaccination.[132] Also, in clinical trials, live JX-594 was detected in throat swabs and skin pustules of patients up to one week after administration.[133] Theoretically, recombination between oncolytic recombinant VV and wildtype VV is possible, however, since VV vaccination is not practiced on a large scale anymore, this is highly unlikely.

In addition, spontaneous mutation rates for VV have been shown to be very low.[134]

## Family Reoviridae: Mammalian Orthoreovirus (mORV)

mORV is a ubiquitous pathogen with high seropositivity in humans, and has been isolated from sewage, stagnant and river water throughout the world.[135,136] mORV is not associated with a named disease in humans, although it can cause mild flu-like upper respiratory or gastrointestinal tract symptoms.[135] Three serotypes of mORV can be distinguished: type 1 Lang, type 2 Jones and type 3 Abney or Dearing (mORV-T3D). mORV-T3D was isolated from the intestinal tract of a child with diarrhea, and is used most in (pre)clinical oncolytic virus research.[137]

mORV-T3D replicates in cells with dysfunctional cell signaling cascades, most importantly (but not exclusively) KRAS-overexpression and subsequent PKR inhibition, making it an inherent oncolytic virus, meaning that it is tumor-specific contributing to safety.[138,139] A multitude of cancer types have been shown to respond to mORV-T3D treatment in (animal) models. Cellular immunity has been found to be important for increasing antitumor efficacy.[137] The absence or inaccessibility of the JAM-A/1 receptor is perceived as a possible limitation for mORV-T3D infection of tumor cells.[140] As such, bio-selection through passaging has been attempted to retarget mORV-T3D to other receptors, although this strategy is probably limited by the quasispecies presence in mORV-T3D isolates.[141] Only one study using recombinant oncolytic mORV-T3D has been described thus far.[142] More studies with recombinant mORV-T3D can be expected in the near future, probably focusing on receptor retargeting and expression of therapeutic or imaging transgenes.

At this time, 16 clinical trials employing intratumoral or intravenous injection of wildtype mORV-T3D (REOLYSIN®: pelareorep) have been conducted and more are currently underway or planned to start in the near future. As excellently summarized by Harrington *et al*. and Maitra *et al*,[137,143] these trials have shown safety with regards to toxicity of mORV-T3D administration to patients with various solid tumors without dose limiting toxicities, while having some appreciable anti-tumor effects in phase II/III trials.

High mORV titers injected intravenously have been shown to be reasonably safe with low toxicity, even in combination with standard therapies like chemo- or radiotherapy, as well as in combination with transient immune suppression.[137,143] Limited mORV shedding has been observed in clinical trials in patient samples of urine, saliva and feces, mostly with high intravenous administrations.[137] As an RNA virus with a viral RNA polymerase, mORV genome replication is prone to errors which can lead to mutations in offspring. Furthermore, since wild-type isolates are in use, these probably represent several quasispecies.[144] Even so, since mORV-T3D does not seem to cause disease in human subjects, the relevance of this mutation rate is low.

## Family Retroviridae: Murine leukemia Virus (MuLV)

MuLVs are widely distributed in domestic and feral mice. MuLVs induce leukemia in mice with latencies ranging from 2 to 18 months, depending on the strain of virus and mouse strain. MuLV is not known to cause a specific disease in humans.[145]

MuLV development for cancer therapy has been focusing on non-replicating, as well as more recently, replication competent retroviral (RCR) oncolytic vectors. The capacity of MuLV and other retroviruses to integrate into the host genome of dividing cells carries the risk of insertional mutagenesis/oncogenesis. Reducing this risk has been an important goal in designing retroviral vectors. The replication capacity of RCR-MuLV is considered to be beneficial for optimizing gene expression in tumors. Recent RCR-MuLV vector genomes consist of an intact viral genome including an IRES-transgene immediately after the stop codon of the *env* gene, which results in more genetic stability, while retaining good replication capacity.[146,147] The fact that RCR-MuLVs can only infect and integrate in dividing cells results in an inherent onco-selectivity. In contrast to most other oncolytic viruses, the oncolytic activity of RCR-MuLVs depends solely on the transgene that is carried by the virus, since infection itself is not cytolytic. To date the transgene of choice has mostly been CD, which converts the antifungal drug 5-fluorocytosine into active chemotherapeutic agent 5-fluorouracil. Oncolytic activity of RCR-MuLV-CD (Toca 511) has been evaluated in preclinical (animal) models for breast cancer, GBM and mesothelioma.[148-150] Toca 511 has a modified backbone and a codon-optimized and heat-stabilized CD gene and has been shown to be highly genomically stable while maintaining oncolytic efficacy upon passaging.[145,149]

Toca 511 is being investigated in clinical trials in the United States in subjects with recurrent high-grade glioma. Up to now, over 70 patients have been treated without dose limiting toxicity and with evidence of clinical oncolytic efficacy.[145] Since RCR-MuLV vectors are capable of genomic integration, germline transmission is a theoretical risk of these vectors, and should be taken into consideration when designing clinical trials.

## Discussion

The field of oncolytic virus research has seen a tremendous progression of several first and second generation vectors toward clinical trials. Most current strategies used in oncolytic virotherapy focus on the use of second and third generation of more virulent conditionally replicating viruses, armed with immune stimulating, anti-tumor or tracking transgenes. Also, immune evasion is still sought after to optimize vector delivery. With the first oncolytic virus talimogene laherparepvec now on the break of FDA and EMA approval, we can expect an even greater interest for this relatively young field of oncologic research in the near future.

The newer generation of oncolytic viruses has been evaluated extensively for their efficacy in preclinical trials, and they have

shown to be more effective than first generation vectors on many occasions. Also, ample evidence has been gathered regarding their safety in terms of toxicity in laboratory animals. However, studies focusing on environmental shedding and possible recombination of these new oncolytic agents with wildtype viruses are scarce. This subject seems to be of less interest to oncolytic virus researchers. However, a good oncolytic virus should also be evaluated for environmental safety. This holds true not only from a scientific point of view, but also from a regulatory and public health point of view. Without a thorough environmental risk assessment, new agents will not be accepted by the regulatory agencies like EMA and FDA for marketing as new therapies. Especially with the newer oncolytic agents becoming more virulent and with the possibility of expressing transgenes that alter the nature of the virus, any possibility of environmental shedding and recombination with wild type virus should be excluded. More studies evaluating the environmental safety of promising oncolytic viruses should therefore be conducted and reported. With proper safety evaluations, oncolytic virotherapy is ready to make the next step toward clinical applications.

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No potential conflicts of interest were disclosed.


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