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New viruses for cancer therapy: meeting clinical needs

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Abstract

Early-stage clinical trials of oncolytic virotherapy have reported the safety of several virus platforms, and viruses from three families have progressed to advanced efficacy trials. In addition, preclinical studies have established proof-of-principle for many new genetic engineering strategies. Thus, the virotherapy field now has available a diverse collection of viruses that are equipped to address unmet clinical needs owing to improved systemic administration, greater tumour specificity and enhanced oncolytic efficacy. The current key challenge for the field is to develop viruses that replicate with greater efficiency within tumours while achieving therapeutic synergy with currently available treatments.

The concept of oncolytic virotherapy originates from clinical reports of cancer regression that coincides with natural viral infections[1]. Virotherapy is currently being developed by genetically modifying viruses for the selective infection and destruction of cancer cells[2,3]. Many viruses have specific tissue tropisms that can be exploited as a starting point for preferential infection and replication within the tumour microenvironment, killing cancer cells while replicating and spreading within disease foci. Clinical trials of oncolysis have been performed for decades[4], but virus engineering strategies have only recently been developed to closely monitor virus replication and to address clinically relevant challenges, such as efficient systemic delivery, tight tumour specificity and improved efficacy in combination with current cancer therapies. By exploiting our ever greater understanding of tumour biology (BOX 1), these advances support the clinical translation of many new and diverse viruses that have been rationally designed to have greater safety and efficacy in the clinic[3,5].

Excellent reviews have thoroughly covered the results of current clinical trials of oncolytic virotherapy[3,6]. In this Review, as an introduction to the field, we summarize the most advanced clinical trials for viruses from nine different families that are currently being tested as anticancer therapies[3]. TABLE 1 lists these selected examples and the modifications of the engineered viruses, the routes of administration and the use of combination therapies for each trial. We focus on three points: first, the increasing diversity of viral families that are being developed for oncolysis; second, the notable safety of currently used viruses, which

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has, in many cases, been shown at the highest doses achievable by today's manufacturing processes; and third, the successes that have been achieved using oncolytic viruses that express immunostimulatory transgenes. As a transition to next-generation preclinical viruses, we also highlight the continued clinical need for improved delivery to and replication within systemic tumours, as well as therapeutic synergy with both the immune system and currently available cancer therapeutics.

Virus families have evolved specificities for different cell types, and this natural diversity is being used for therapeutic development. Currently, there are viruses from nine different families in clinical trials: *Adenoviridae*[7], *Picornaviridae*[8], *Herpesviridae*[9,10], *Paramyxoviridae*[11,12], *Parvoviridae*[13], *Reoviridae*[14,15], *Poxviridae*[16–18], *Retroviridae*[19] and *Rhabdoviridae*[20,21] (TABLE 1). Viruses from all of these families (except *Reoviridae* and *Parvoviridae*) have been engineered to have greater tumour specificity and/or efficacy than their parental strains. A reverse genetics system has become available for reovirus[22], which will enable further development of this virus family in the future. Broadened virus availability is exemplified by the newly discovered picornavirus Seneca Valley virus, which was found to preferentially infect neuroendocrine tumours, such as small cell lung cancer[23]. Indeed, robust replication was specifically observed in patients who had small cell lung cancer[8], and this has led to the initiation of a Phase II clinical trial.

Generally, oncolytic virotherapy has been a well-tolerated experimental clinical therapy after both localized and systemic administration[5]. The most common adverse effects are fever and general flu-like symptoms, but more serious toxicities have been documented in rare cases[5,6]. In addition, no transmission of an oncolytic virus from treated patients to carers or other contacts has been noted, although shedding of virus has been documented in the urinary and respiratory tracts, especially after systemic administration. Continuing technological advances in virus production should enable more aggressive dosing in future trials[3], placing greater onus on the tumour specificity of future viruses to maintain current safety profiles.

An important result from clinical trials is the success of therapeutic protocols that are based on the expression of the immunostimulatory cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF). Replicating viruses from three different families (TABLE 1), talimogene laherparepvec (Amgen; *Herpesviridae*), JX-594 (Jennerex Biotherapeutics; *Poxviridae*) and CG0070 (Cell Genesys; *Adenoviridae*), combine replicative onco lysis with GM-CSF-mediated stimulation of granulocytes and monocytes to induce inflammation and adaptive immunity against tumour antigens. All three viruses have shown efficacy in advanced clinical trials: the results of a Phase II trial for talimogene laherparepvec in melanoma have been reported[9] and a Phase III trial in melanoma has recently been completed; JX-594 has been tested in a randomized Phase II trial in hepatocellular carcinoma[18]; and CG0070 is currently being tested in a Phase II trial for bladder cancer[24].

Although Phase I and II clinical trials are not designed to directly measure efficacy, most current-generation viruses in clinical trials have fallen short of the efficacy expectations that were set by preclinical models[2,3]. Improving efficacy is a multifactorial challenge, and in this Review, we summarize the preclinical engineering strategies for the virus families that

have been most extensively studied so far. We describe engineering strategies that focus on overcoming continued clinical challenges: resisting antibody neutralization using genetic and chemical virus shielding; improving tumour specificity by targeting tumour-associated receptors and controlling post-entry viral replication; and improving therapeutic synergy with the immune system, chemotherapy and radiotherapy. By carefully pairing diverse virus families with engineering strategies and combination therapies, next-generation viruses can be rationally designed for greater efficacy against diseases that have unmet clinical needs.

Avoiding virus neutralization

Systemic administration of oncolytic viruses via the vasculature gives the virus access to all perfused regions of primary and metastatic tumours, making it the preferred administration route for the treatment of metastatic disease. However, systemic delivery also makes the virus susceptible to inactivation by pre-existing antibodies in the blood, which can arise in patient populations via natural contagion, scheduled immunization or prior administration of a therapeutic virus. Pre-existing and induced antiviral immune responses can be pharmacologically tempered to limit the neutralization of therapeutic viruses[25–29]. In this section, we discuss virus engineering strategies that can shield therapeutic viruses from pre-existing neutralizing antibodies by changing or physically masking the epitopes that are recognized by the antibodies. Multiple engineering strategies that differ in the type and magnitude of modification have been applied to different virus families, but the end result is always a chimeric virus with an engineered serotype that is not recognized by the pre-existing antibodies present in the target patient population (FIG. 1). We use vesicular stomatitis virus (VSV), adenovirus and measles virus to illustrate these different strategies, highlighting both the type and the extent of each modification, as well as discussing the applicability of these strategies to other virus families and combination regimens with pharmacological immunosuppression.

Serotype exchange

The most basic method of genetic shielding is serotype exchange, whereby a different serotype of the same virus species is engineered onto the core of an established virus, generating a chimeric virus that has the donor serotype but the original core (FIG. 1a). This strategy requires the availability, for a given virus, of multiple serotypes, which typically have less than 60% identity between their surface-exposed glycoproteins or capsid proteins[30]. For example, all neutralizing epitopes that are present on VSV particles are found in the VSV-G glycoprotein, therefore serotype switching can be accomplished by simply replacing the entire VSV-G glycoprotein gene sequence with that of another serotype (FIG. 1a). Serotype switching for adenovirus is more complicated because multiple surface-exposed capsid proteins, such as the hexon or fibre knob proteins, contain neutralizing epitopes, as recently reviewed in REF. 31. The hexon capsid protein encodes seven distinct hypervariable regions that all contribute to determining the serotype of the virus[32]; by modifying all seven hypervariable regions, the human adenovirus 5 (HAdV-5) serotype, against which most of the population has neutralizing immunity, can be substituted for the much rarer HAdV-48 serotype[33] (FIG. 1b). Replacing the HAdV-5 fibre knob with that from HAdV-3 also generated a less immunogenic chimeric virus that was resistant to neutralization by serum

from HAdV-5-immunized mice during gene transfer *in vivo* and *ex vivo*[34] (FIG. 1b). However, in addition to changing serotypes, knob modifications can change receptor specificity: replacing the fibre of HAdV-5 with the fibre from HAdV-16 or HAdV-50 changes receptor specificity from coxsackie–adenovirus receptor (CAR) to the ubiquitous CD46 molecule[35]. Notwithstanding tropism modifications, which are discussed below, these genetic exchanges can create viruses that have unique serotypes built around a common viral backbone.

Novel serotypes

A variation of the serotype exchange strategy has been implemented for monotypic viruses, such as measles virus, which have only one serotype. The measles virus glycoproteins were replaced with those of canine distemper virus (CDV), which is also a *Morbillivirus*, to generate an infectious virus that is not neutralized by anti-measles virus antibodies[36] (FIG. 1c). Importantly, CDV and measles virus, and possibly all members of the *Morbillivirus* genus, enter cells using the same primary receptors — signalling lymphocytic activation molecule (SLAM) and nectin 4 — although there are differences in cross-species receptor recognition[37,38]. The applicability of glycoprotein exchange is limited by the availability of suitable envelope donors that are compatible with measles virus; the glycoproteins from a closely related virus of another genus did not sustain efficient assembly of chimeric particles[39]. Much like serotype exchange, glycoprotein or capsid exchange between viruses requires a balance between protein diversity that is sufficient for avoiding cross-neutralization and sequence and structural similarities that are necessary to support particle formation.

Chemical modifications

Shielding strategies can be used to generate a repertoire of viruses that are built around a common core but that have unique serotypes. Such a repertoire can then be used for sequential rounds of therapy that maintain efficacy even as patients develop immunity to previously used viruses. This sequential administration strategy using engineered serotypes can enhance efficacy by expanding the therapeutic window for virotherapy and, at the same time, maintaining common targeting and arming strategies.

Chemical modifications of virus particles help to overcome some of the challenges that are associated with genetic shielding. Polymer deposition on particles can physically shield epitopes from antibody neutralization (FIG. 1d); polyethylene glycol (PEG) and poly-*N*-(2-hydroxypropyl) methacrylamide (poly-HPMA) polymers are two examples of polymers that are used to shield oncolytic viruses[40,41]. Chemical shielding has been most extensively applied to *Adenoviridae* to protect particles from inactivation in the blood and decrease off-target liver transduction[42–45], but other virus families, such as *Rhabdoviridae*[46] and *Poxviridae*[47], have also been chemically modified. The utility of chemical shielding depends on viruses retaining their ability to enter cells after polymer deposition, which has been successful with the icosahedral adenovirus and enveloped viruses, such as VSV and vaccinia virus. Alternatively, polymer shields that are linked to specific ligands can be used to restore and target virus entry[41,48], as discussed below. Importantly, chemical shielding strategies

simplify virus production, but *in vivo* virus replication will leave progeny particles unprotected, potentially limiting efficacy in applications that depend on virus spread.

Tumour targeting

In this Review, we use the term targeting to describe virus modifications that confer greater specificity for tumour cells by improving infection of diseased tissues and decreasing infection of healthy tissues. This specificity can be enhanced either at the stage of virus entry into target cells or post-entry during replication. Entry targeting can be achieved by fusing or conjugating specificity domains that modify receptor usage of virus particles. Post-entry, tumour-specific replication targeting, using promoters and engineered microRNA (miRNA) target sequences, can restrict virus replication in off-target tissues. These targeting strategies differ fundamentally from those that were used in the first-generation oncolytic viruses, which were often based on the removal of virulence factors that are redundant for replication in tumours[49]. Although this enhances safety in normal tissues, it often limits replicative fitness in target tissues[50]. Instead, these novel retargeting strategies can be applied to viruses that have more wild-type characteristics to minimize off-target toxicity without compromising virus replication within disease foci.

All viruses require interactions with surface molecules on host cells to start infection. Viruses can be specifically retargeted to recognize molecules that are preferentially or exclusively expressed on tumour cells (BOX 1). This strategy requires modifications to be made to the receptor-binding proteins that are present on viral particles. These modifications are either genetic and generate chimeric proteins, or they use chemical adaptors to link specificity domains to virus particles[51]. Targeting of enveloped viruses from the *Paramyxoviridae* and *Herpesviridae* families has rapidly progressed owing to the plasticity of their glycoproteins and the separation of receptor-binding and membrane-fusion functions, which are mediated by different proteins[52]. By contrast, non-enveloped icosahedral viruses, such as the *Adenoviridae*, have very stringent structural constraints on particle assembly, which limit viable modifications to short peptides or the chemical retargeting of assembled particles[44,53] (FIG. 2).

Receptor targeting: Paramyxoviridae

Among enveloped viruses, *Paramyxoviridae* remain the preferred platform for the development of new targeting strategies because receptor binding and membrane fusion are mediated by two different proteins, the attachment protein (haemagglutinin, haemagglutinin-neuraminidase or glycoprotein) and the fusion protein, respectively. Adding specificity domains, such as single-chain antibodies, to the carboxyl terminus of the attachment protein sustains binding to designated receptors and subsequent membrane fusion is achieved by the unmodified fusion protein (FIG. 1). Recombinant measles virus particles that express a retargeted haemagglutinin in place of the standard haemagglutinin can be reliably generated and stably passaged[54]. In addition, entry via the natural receptors can be ablated by mutating specific haemagglutinin protein residues that are necessary for binding and/or entry[55]. This targeting principle has recently been extended to designed ankyrin repeat proteins (DARPins), which are engineered repeat-motif proteins that are smaller than single-chain antibodies and can be combined to achieve multiple specificities[56]; for example, it was

shown that DARPins can simultaneously retarget measles virus to two different tumour markers[57]. This dual retargeting strategy could theoretically be used to target both tumour parenchyma (for debulking) and $CD133^+$ cancer-initiating cells (for prolonged growth inhibition[58]) using a single virus, as well as to safeguard against tumour resistance due to heterogeneous or downregulated receptor expression.

Receptor targeting: Herpesviridae

The herpes simplex virus (HSV) entry mechanism is more complex than that of the *Paramyxoviridae*. HSV relies on five proteins, glycoprotein C, glycoprotein B, glycoprotein D and the glycoprotein H–L dimer, for receptor binding and membrane fusion, which can occur at the plasma membrane or in endocytic vesicles[59,60]. Glycoprotein D is responsible for binding three different receptors: the herpesvirus entry mediator (HVEM); the cell adhesion molecule nectin 1; and 3-*o*-sulphotransferase-modified heparan sulphate[59,60] (FIG. 2). Despite this complexity, HSV retargeting shares common themes with measles virus: glycoprotein D can be engineered to express ligands, such as interleukin-13 (IL-13)[61] or urokinase plasminogen activator[62,63], or single-chain antibodies against human epithelial growth factor receptor 2 (HER2; also known as ERBB2)[64] near its amino terminus, which retargets HSV to antigens expressed on gliomas and breast tumours, respectively. Furthermore, the natural receptor tropism can be ablated by sterically blocking the receptor-binding interfaces of glycoprotein D with a single-chain antibody[64] or by using a single chain antibody to replace the entire imunoglobulin core of the glycoprotein[65], which results in simultaneous retargeting of the virus to HER2 and detargeting from its natural receptors. In addition, glycoprotein C functions with glycoprotein B during initial cell binding and can be used to retarget entry using small deletions and appended single-chain antibodies[66]. Modifications to glycoprotein B and glycoprotein D have also been combined: entry-accelerating mutants of glycoprotein B have been paired with single-chain variable fragment (sc-Fv)-engineered envelope glycoprotein D to improve the efficiency of HSV retargeting to epidermal growth factor receptor, which is expressed on glioblastomas[67]. Although the entry mechanism of HSV is more complex than that of measles virus, both viruses can be genetically engineered for targeted entry using similar strategies.

Receptor retargeting: Adenoviridae

Adenovirus entry targeting is more demanding than that of *Paramyxoviridae* or *Herpesviridae* owing to the constraints of the icosahedral particle structure. Nevertheless, short heterologous peptides and specificity domains that recognize tumour-associated antigens have been inserted into the HI loop and the C terminus of the receptor-binding trimeric fibre protein, which is located at the vertices of the capsid[53,68–71]. The HI loop, in particular, is flexible and the inserted short peptides have minimal negative effects on virus fitness, even in combination with mutations that detarget viruses from natural adenovirus receptors[68]. In addition, an adenoviral minor capsid protein, the hexon-interlacing protein IX (also known as `cement' protein IX), can be used to successfully retarget entry using specificity domains, such as single-domain antibodies that do not require oxidation for folding, but not domains that are folded in the endoplasmic reticulum, such as single-chain antibodies[72]. A second strategy for adenovirus retargeting uses adaptors to non-covalently link particles to larger specificity domains[44,53] (FIG. 1): X-ray crystallography structural

data were recently used to develop universal adaptors that have high affinity for adenovirus fibre. These adaptors were then linked to DARPin specificity domains to retarget the cell entry of coated particles[73]. Such a universal adaptor makes it possible to target many different tumour markers using a single starting virus but, as discussed above for shielding, chemical virus retargeting is limited to a single round of replication.

Post-entry targeting

In this section we consider two post-entry targeting principles: positive targeting — which selectively promotes the expression of viral genes or engineered transgenes in target cells using tumour-specific transcriptional control[74,75] — and negative targeting — which restricts infection in non-target cells using tissue-specific miRNAs that recognize target sequences that have been engineered into oncolytic virus genomes (FIG. 3). These strategies are complementary and are being applied, sometimes in combination, to multiple virus families (FIG. 2).

Tumorigenesis is, in part, driven by aberrantly high transcription levels of genes that are not expressed in normal tissue (BOX 1). Positive transcriptional targeting can control the expression of virus genes as they have been determined to be dependent on overexpressed, tumour-specific promoters[76] (FIG. 3a). Positive transcriptional targeting has been most extensively applied to adenovirus using differentially expressed tumour-associated promoters, such as telomerase (reviewed in REFS 77–79). Novel tumour-associated promoters can be identified for individual diseases using gene expression profiling, for example, by comparing transcriptional profiles between hepatocellular carcinoma (HCC) and normal liver[76]. This strategy has been used to identify and validate HCC-specific promoter expression *in vitro* and in HCC xenografts in mice[76], and then to exploit those promoters to activate the expression of wild-type virulence factors, such as HSV-infected cell protein 27 (ICP27) and ICP34.5, specifically in diseased tissues[80,81]. Positive replication targeting can be applied to any virus family that relies on the cellular machinery for transcription but not to viruses that use virally encoded polymerases for replication in the cytoplasm, such as measles virus and vaccinia virus (FIG. 2).

An alternative strategy for regulating replication is the insertion of miRNA target sequences within the untranslated regions (UTRs) of virus transcripts to provide negative post-transcriptional regulation in non-target tissues (FIG. 3 b). Proof-of-principle for this targeting strategy was first shown using an oncolytic picornavirus that had miRNA target sequences for muscle-specific miRNA in its genome. Indeed, virus replication in muscles was minimal owing to the cellular miRNA machinery recognizing and degrading viral transcripts, thereby eliminating toxic myositis without negatively affecting viral oncolysis[82]. Negative replication targeting is versatile and has been applied to *Adenoviridae*[83–88], *Herpesviridae*[89,90], *Paramyxoviridae*[91], *Poxviridae*[92] and *Rhabdoviridae*[93]. The common principle is to express perfect-match miRNA target sequences, often multiple sequences in tandem, in the UTRs of essential viral genes. These target sequences are chosen on the basis of abnormally low expression of specific miRNAs in tumours. With increasing knowledge about miRNA expression in tumours[94,95] (BOX 1) and the relative ease with which these

target sequences can be incorporated into virus genomes without negatively affecting replication, this tumour-targeting strategy has broad applicability (FIG. 2).

Combined post-entry targeting

Positive and negative replication targeting have recently been combined to create an HSV virus that depends on the liver-specific apolipoprotein E–α-1-antitrypsin (AAT) promoter for the expression of envelope glycoprotein H and is restricted in normal liver (but not in tumours) by three differentially expressed miRNAs[90]. This combination of positive and negative targeting effectively blocks replication of this virus in tissues other than hepatic tumours without the need to remove the virulence factors that are necessary for optimal oncolytic efficacy. Oncolytic adenovirus has also been dually targeted using positive and negative replication engineering to restrict virus infection in normal liver but not in multiple tumour types[85]. When combined, these targeting strategies effectively limit virus replication to tumour cells, even if promoter and miRNA expression are insufficient for tight restriction alone. As next-generation viruses that have more wild-type characteristics are developed, it will be possible to counteract their potential for greater toxicity using combinations of entry and post-entry retargeting strategies that are applicable to many different virus families.

Arming

Oncolytic viruses must infect and kill tumour cells to achieve efficacy, and despite intratumoural replication, accessing and infecting 100% of tumour cells remains a major clinical challenge for therapeutic viruses. Therefore, the therapeutic efficacy of oncolytic viruses can be enhanced using strategies that induce `bystander cell killing', whereby a protein that is expressed by the oncolytic virus sensitizes both the infected cell and surrounding uninfected cells to subsequent combination therapies or immune destruction. Prodrug convertases that are expressed from oncolytic viruses can enhance the efficacy of chemotherapy by activating prodrugs, ion transport proteins can promote radiation poisoning of tumours owing to the concentration of radioisotopes, and immunostimulatory factors can induce innate and adaptive immune responses to tumour-associated antigens (FIG. 4). We discuss these three types of virus arming below.

Prodrug convertases

Prodrug convertases include the thymidine kinase[96,97], the cytosine deaminase[97] and the purine nucleoside phosphorylase (PNP)[98] systems. The corresponding genes have all been expressed by oncolytic viruses to activate non-toxic precursors, which generate highly toxic metabolites in the tumour microenvironment[99]. The HSV thymidine kinase phosphorylates ganciclovir to generate ganciclovir triphosphate, cytosine deaminase converts chemotherapeutic 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), and PNP converts fludarabine phosphate into 2-fluoroadenine[99]. These nucleoside analogues are incorporated into the DNA of replicating cells, which halts replication and ultimately results in cell death[99]. The common goal of these strategies is to reduce systemic toxicity by giving lower doses of minimally toxic chemotherapeutic drugs that are only converted to highly toxic metabolites within the tumour microenvironment (FIG. 4a). Ganciclovir triphosphate cannot easily diffuse between cells after activation[100], making it a good option for the selective

elimination of the toxicity that is induced by retrovirally transduced cells[101,102]. However, the activated metabolites 5-FU and 2-fluoroadenine can both diffuse out of the infected cell and into surrounding cells[98,103] to induce chemotherapeutic bystander killing (FIG. 4a), making them clinically relevant arming strategies for virotherapy. We focus in this section on the most clinically advanced viruses that are armed with the cytosine deaminase and PNP transgenes, as well as on promising preclinical viruses that are ready to enter future clinical trials.

Cytosine deaminase and PNP have both been incorporated into several virus classes that have been preclinically tested, including viruses that are based on *Herpesviridae*[104], *Adenoviridae*[105], *Poxviridae*[106,107], *Paramyxoviridae*[108,109] and *Rhabdoviridae*[110]. The most advanced cytosine deaminase virus is a replication-competent retrovirus known as Toca 511, which integrates the cytosine deaminase transgene into the genome of infected cells to establish permanent reservoirs of tumour cells that are sensitive to subsequent rounds of chemotherapy using 5-FC[19]. Toca 511 is currently being tested in combination with 5-FC in Phase I and II clinical trials using intratumoural administration in patients with grade 4 glioblastoma multiforme (TABLE 1). HSV and VSV viruses that express cytosine deaminase are also being preclinically developed for combination therapies using 5-FC[104,110].

Intratumoural administration of an adenovirus with the PNP transgene in combination with intravenous fludarabine has been used to treat patients with head and neck tumours[111] (clinicaltrials.gov identifier: NCT01310179). In general, cytosine deaminase and PNP transgenes can be applied to many virus families because their small sizes and low cellular toxicities incur minimal negative effects on *in vitro* virus fitness or production. However, the timing of prodrug dosing *in vivo* must be optimized to ensure that virus replication and spread is sufficient for maximal synergistic effects with the chemotherapeutic prodrug[104,109,112]. In this respect, a PNP-expressing measles virus that has been retargeted to CD20 has been extensively tested in combination with fludarabine in preclinical models of lymphoma[108,109].

Radiosensitization

The normal physiological function of the human sodium–iodide symporter (NIS) is to transport iodide ions into cells, which occurs predominantly in the thyroid but also in the stomach, salivary glands and mammary glands[113,114]. When NIS is expressed from the genome of an oncolytic virus, infected cells concentrate iodide or similar isotopes intracellularly. During virotherapy, γ-emitting isotopes, such as ^{123}I and pertechnetate, can be administered to visualize virus replication using single-photon emission computed tomography (SPECT; BOX 2), whereas β-emitting isotopes, such as ^{131}I and ^{188}Re, can be administered to specifically induce radiation poisoning within the tumour microenvironment (FIG. 4b), in analogy to the clinically well-established radiotherapy that is used for metastatic thyroid cancer.

The NIS transgene system has undergone extensive preclinical development in multiple virus families, and radiovirotherapy has consistently achieved synergistic tumour destruction in several radiosensitive preclinical disease models[115–117]. Measles virus is an especially

efficacious virus for NIS-mediated imaging (BOX 2) and radiovirotherapy. Preclinical studies of lymphoma[118], ovarian cancer[119], myeloma[120] and mesothelioma[121], in addition to many other disease models[117], have used NIS expression and SPECT–computed tomography (SPECT–CT) imaging to visualize and quantify virus replication and enhance disease regression using combination radio virotherapy. Phase I clinical trials using NIS-expressing measles virus have been initiated for ovarian cancer, myeloma, mesothelioma and head and neck cancer (clinicaltrials.gov identifiers: NCT00408590, NCT00450814, NCT01503177 and NCT01846091, respectively), with all four studies using SPECT–CT intervention to image virus replication in patients. The results of these clinical trials and the continued translation of diverse NIS-expressing viruses in different tumour types will inform the applicability of SPECT–CT imaging and radiovirotherapy interventions to future trials.

Immunostimulation

Advanced clinical trials of oncolytic viruses from three different virus families have used the combination of lytic infection and immunostimulatory transgene expression to induce anti tumour immunity (FIG. 4c). The most successful strategy that has been used so far is the expression of GM-CSF to stimulate the production of granulocytes and monocytes, which in turn stimulate adaptive immunity against tumour-associated antigens[122]. HSV, adenoviruses and vaccinia viruses that express GM-CSF are currently used in clinical trials and have repeatedly been shown to have clinical efficacy [122,123], especially in diseases that are amenable to immunotherapy, such as melanoma (BOX 1). These advanced clinical studies have shown that these viruses work via immunotherapy rather than via virus-mediated tumour lysis: only moderate replication of vaccinia JX-594 was documented in tumour biopsies or measured by the detection of GM-CSF in the blood, but lytic replication or recovery of infectious virus from harvested tumours were not reported[18,124]. The HSV virus talimogene laherparepvec[9,125] is currently being tested against melanoma in a Phase III clinical trial that will directly compare the virus expressing GM-CSF with GM-CSF administration alone. This study will greatly improve our understanding of the relative contributions of virus replication and transgene expression in stimulating antitumour immunity. The successes of current oncolytic viruses that express immunostimulatory transgenes, even in the absence of robust intratumoural replication, highlights the potential for new viruses that have greater replication and transgene expression to stimulate improved, and potentially curative, immune responses against the tumour microenvironment.

Future directions

Our increasing knowledge of the determinants of virus tropism, and of the proteins and gene expression pathways that are altered in tumour tissues, are important resources for developing next-generation viruses. With few exceptions, such as vaccinia virus and VSV, virus families have adapted to tissue niches, which are often defined by specific receptors; for example, measles virus initially uses SLAM to establish systemic infection in lymphatic organs, and then nectin 4 to infect epithelia[126–129]. Oncolysis, similarly to wild-type virus spread, depends not only on efficient cell entry via specific receptors, but also on efficient replication; in particular, viruses often activate or exploit certain gene expression pathways that facilitate their replication, and similar events may be required for efficient oncolysis.

Consequently, efficient cell entry might not always result in efficient oncolysis; for example, measles virus-based oncolytic viruses are likely to replicate most efficiently in SLAM-positive haematological malignancies, such as lymphoma, and in nectin 4-positive epithelial malignancies, such as breast[130], ovarian[131] and lung[132] tumours. As there is an increasingly diverse pool of oncolytic viruses and our knowledge of tumour biology is improving, choosing virus classes to target specific tumour types, and then stratifying individual patients on the basis of disease susceptibility to virotherapy, should soon become standard practice.

Oncolytic virotherapy has, to date, proven to be very safe in humans. By contrast, the therapeutic efficacy of oncolytic viruses in humans has been less than expected from preclinical studies. Early protocols were based on highly attenuated viruses, and generalized attenuation interfered with clinical efficacy. Next-generation viruses will benefit from retaining wild-type replicative potential in disease tissue, combined with engineered entry and post-entry restriction mechanisms that maintain current safety profiles in off-target tissues. In addition, arming strategies that combine chemo-, radio- and immuno-therapies will be potentiated by greater virus replication, and advances in *in vivo* imaging will enable real-time tracking of virus spread (BOX 2).

New knowledge and new viruses can now be used to address existing clinical needs. Although many challenges remain for oncolytic virotherapy, such as the production and validation of an increasing number of therapeutic viruses, there are strategies to overcome them. There is no single best virotherapy approach for all tumours, but creative engineering strategies that use viruses from diverse families are yielding new viruses that are likely to be highly efficacious in specific applications. Understanding the susceptibility and resistance of different tumour types and progress in stratifying individual patients will facilitate the recruitment of those patients who are most likely to benefit from a new virus. In synergy with approved cancer therapies and antitumour immunity, these new viruses are expected to achieve the clinical promise of oncolytic virotherapy.

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Glossary

Granulocyte–macrophage colony-stimulating factor	(GM-CSF). An immunostimulatory cytokine that functions as a growth factor for granulocytes and monocytes
Serotype	A collection of viruses grouped according to common antigenic epitopes that are recognized by the same immune serum

Coxsackie–adenovirus receptor	(CAR). An immunoglobulin-like transmembrane cell adhesion protein that is used by some coxsackievirus and adenovirus species as a receptor
CD46	A ubiquitously expressed type-1 transmembrane protein that functions to regulate complement. It functions as a receptor for vaccine strains of measles virus and some adenovirus species
Parenchyma	The bulk of tumours; consists of clonally transformed cells
$CD133^+$ cancer-initiating cells	A subset of tumour cells that have stem cell-like characteristics. They are capable of initiating new tumour growth and may drive tumour recurrence after therapy
Prodrug	A chemotherapeutic drug that is dependent on enzymatic activation to achieve full activity
Radioisotopes	Elements with unstable nuclei that emit γ-rays or α- or β-ionizing radiation
Nucleoside analogues	Chemotherapeutic drugs that incorporate into replicating DNA as nucleosides but halt DNA replication, which leads to cell death
γ-emitting isotopes	Radioactive isotopes that emit ionizing radiation in the form of high-energy electromagnetic γ rays
β-emitting isotopes	Radioactive isotopes that emit ionizing radiation in the form of high-energy, high-speed electrons or protons

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Box 1 | Targeted oncolysis: exploiting improved understanding of tumour biology

Genetically modified viruses can exploit different classes of tumour-specific abnormalities for efficient and specific oncolysis. First, tumour targeting can take advantage of the preferential expression of certain proteins on the cell surface; these proteins can be repurposed as receptors for virus attachment and cell entry. Second, promoters and enhancers that are particularly active in tumour cells can be used to drive the expression of certain viral genes, thereby governing viral replication. Third, viral gene expression can be made more tumour-specific by inserting sequences that are complementary to endogenous microRNAs (miRNAs) into viral genomes. Fourth, tumour-associated antigens can become immunogenic if they are exposed to the immune system during viral infection and in the context of immunostimulatory transgene expression.

Most first-generation oncolytic viruses targeted only one of these tumour-specific characteristics, but most viruses that are currently in preclinical trials target two or more simultaneously. These developments are made possible by our improved understanding of tumour biology, which is reflected by the availability of databases that profile different tumour characteristics; for example, databases of microarray expression data, such as the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) can be used to identify both the transcription levels of surface proteins and the promoter activity abnormalities that are specific to diseases of interest. Transcriptome profiling tools, such as RNA-sequencing[133] and the Encylcopedia of DNA Elements (ENCODE) database[134], can be used to put these data in the context of transcription and protein expression in normal tissues. Tumour-specific miRNA sequences can be queried in databases, such as the miRNA database (miRBase) and microRNA.org, and Cancer Genome Atlas researchers are mapping the genetic changes in 18 different types of cancer.

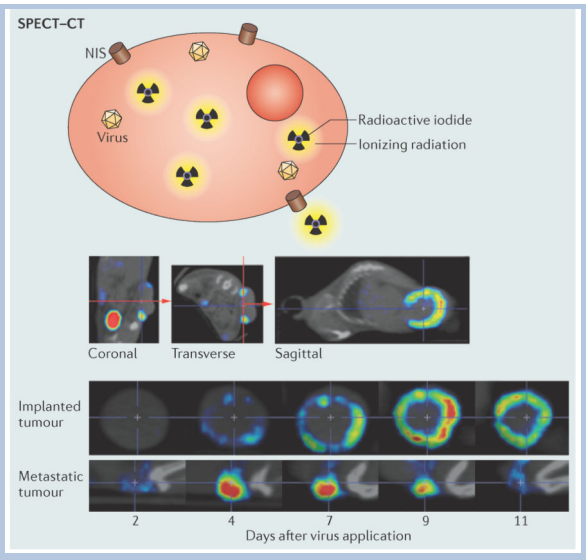
As the molecular pathophysiology of different diseases is characterized[135], quantitative insights will emerge about the frequency with which different tumour-associated antigens are detected in patient populations. These insights will enable recombinant viruses that have broad oncolytic activities to be used for the treatment of specific diseases. Moreover, gene expression profiles from patients will help to identify those individuals that have the highest probability of responding well to therapy with a specific virus.

Box 2 | *In vivo* imaging

The optimization of virotherapy, in particular the arming strategies that are outlined in this Review, generally requires the characterization of virus replication in target tissues and an understanding of its determinants. Single-photon emission computed tomography (SPECT) and positron emission tomography (PET) are clinically relevant imaging approaches that are capable of achieving impressive resolution in small animal models and in humans. These imaging modalities rely on transgenes, such as the sodium–iodide symporter (NIS), that cause infected cells to accumulate radioactive tracers that have detectable emissions. Some tracers, including those that emit γ-radiation, have more effective tissue penetration than fluorescence or bioluminescence. Only effective tissue penetration enables whole-body analyses to be performed in the clinic.

Dedicated imaging systems have been developed for small animals, and recent advances in SPECT imaging have achieved submillimetre resolutions for whole-animal tomographic analyses. The figure shows an *in vivo* analysis of viral replication by SPECT–computed tomography (SPECT–CT) imaging as an example. NIS, expressed from an oncolytic measles virus, induces isotope accumulation within infected cells. The top row of images illustrates how the base of the implanted tumours is aligned in the coronal and transverse planes to obtain three-dimensional tumour images with equivalent orientation at different times. The central and bottom rows of images document virus replication and show a ring being formed in the outer region of the implanted tumour by day 9 after virotherapy (middle row), whereas infection and replication in a metastatic tumour are more intense and peak earlier (bottom row). Thus, SPECT–CT imaging allows four-dimensional (three spatial dimensions plus time) analyses of virus spread in tumours in living hosts. SPECT–CT, as well as advanced PET and magnetic resonance imaging (MRI) strategies that are excellently reviewed in REFS 136–138, are broadly applicable to many oncolytic virus families and can inform sequential rounds of therapy, correlate virus replication and distribution with measures of clinical efficacy and, in worst-case scenarios, can identify off-target virus replication.

SPECT–CT images are reproduced, with permission, from ref. 118 © (2013) American Society for Gene and Cell Therapy (ASGCT). High-resolution videos of SPECT–CT data can be viewed in ref. 118.



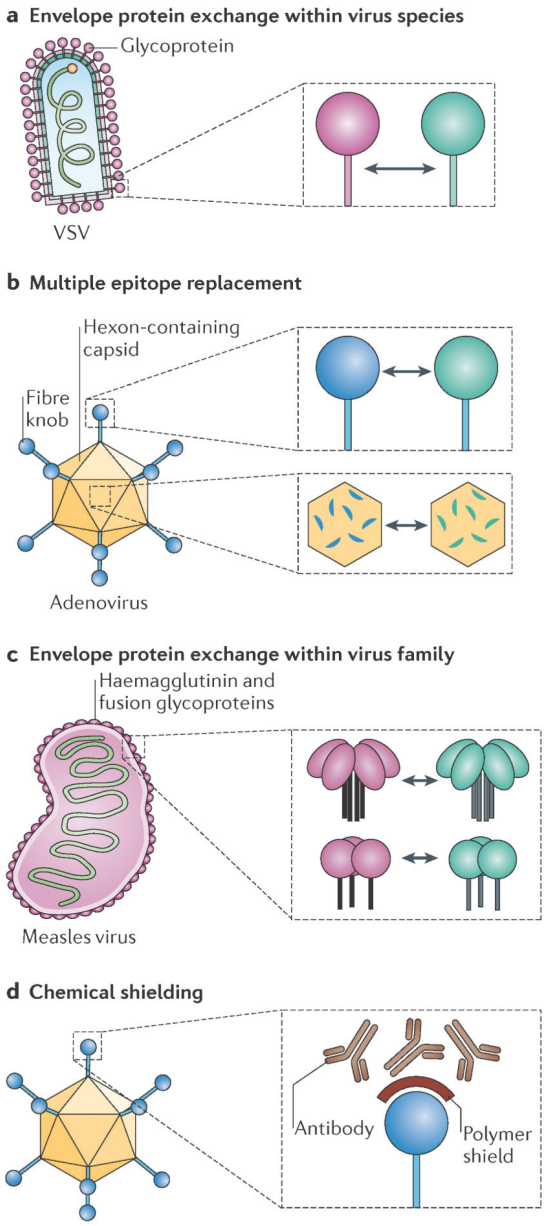


Figure 1. Vector-shielding strategies

a | Interchange of different serotypes from the same virus species is shown for vesicular stomatitis virus (VSV), which has only one envelope glycoprotein. **b** | Replacement of multiple immunogenic epitopes in different proteins is shown for adenovirus; exchange can be achieved using full domains (such as the fibre knob) or individual motifs (such as hexon hypervariable loops). **c** | Generation of a new serotype is shown for measles virus. The two glycoproteins of this monotypic virus are substituted by the glycoproteins of an animal virus of the same genus. **d** | Chemical shielding of viral epitopes is shown for adenovirus; small polymers can be added to purified viral particles.

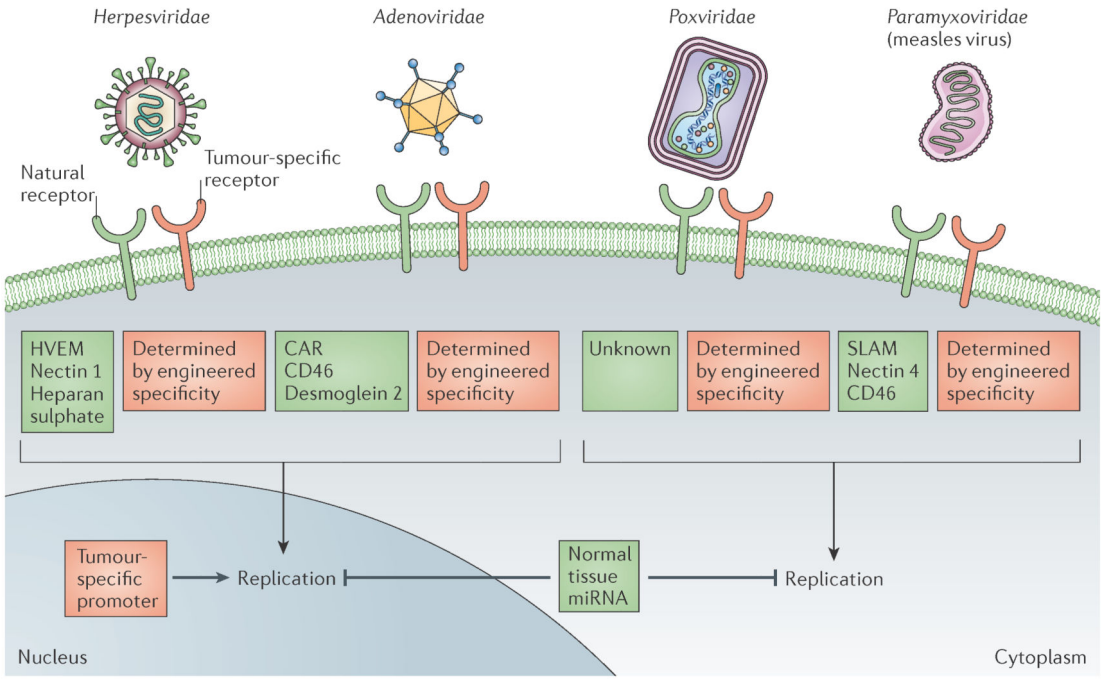


Figure 2. Principles of tumour targeting — illustrated for four virus families

From top to bottom: targeting cell entry (detargeting from natural receptors and retargeting to tumour surface markers) and post-entry targeting (targeting of transcription, replication or microRNAs (miRNAs)). CAR, coxsackie–adenovirus receptor; HVEM, herpesvirus entry mediator; SLAM, signalling lymphocytic activation molecule.

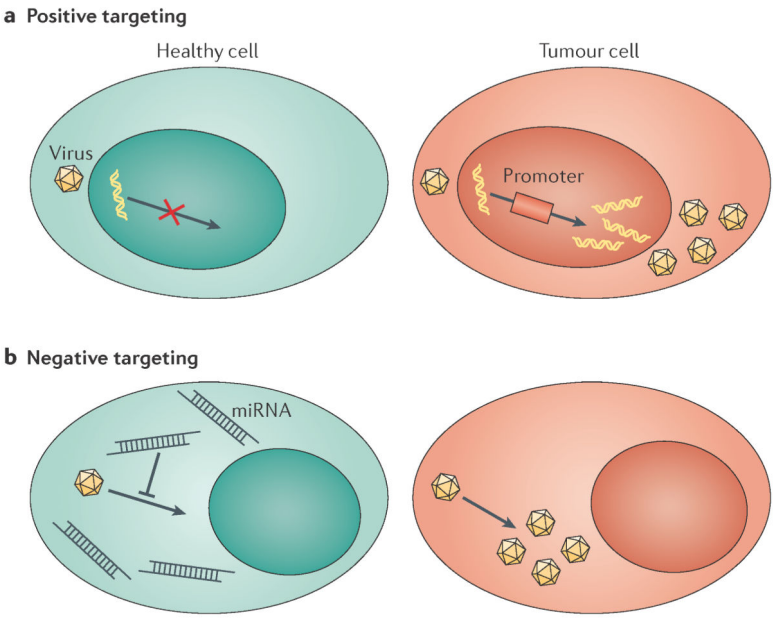


Figure 3. Post-entry targeting

a | Positive transcription targeting relies on promoters that are highly expressed in cancer cells to stimulate the preferential expression of viral genes or transgenes in tumours. **b** | Negative targeting depends on microRNA (miRNA) expression in normal cells to restrict the replication of vectors that express miRNA-recognition sequences within their genomes. Tumours have decreased expression of certain miRNAs, which renders them unable to restrict vector replication.

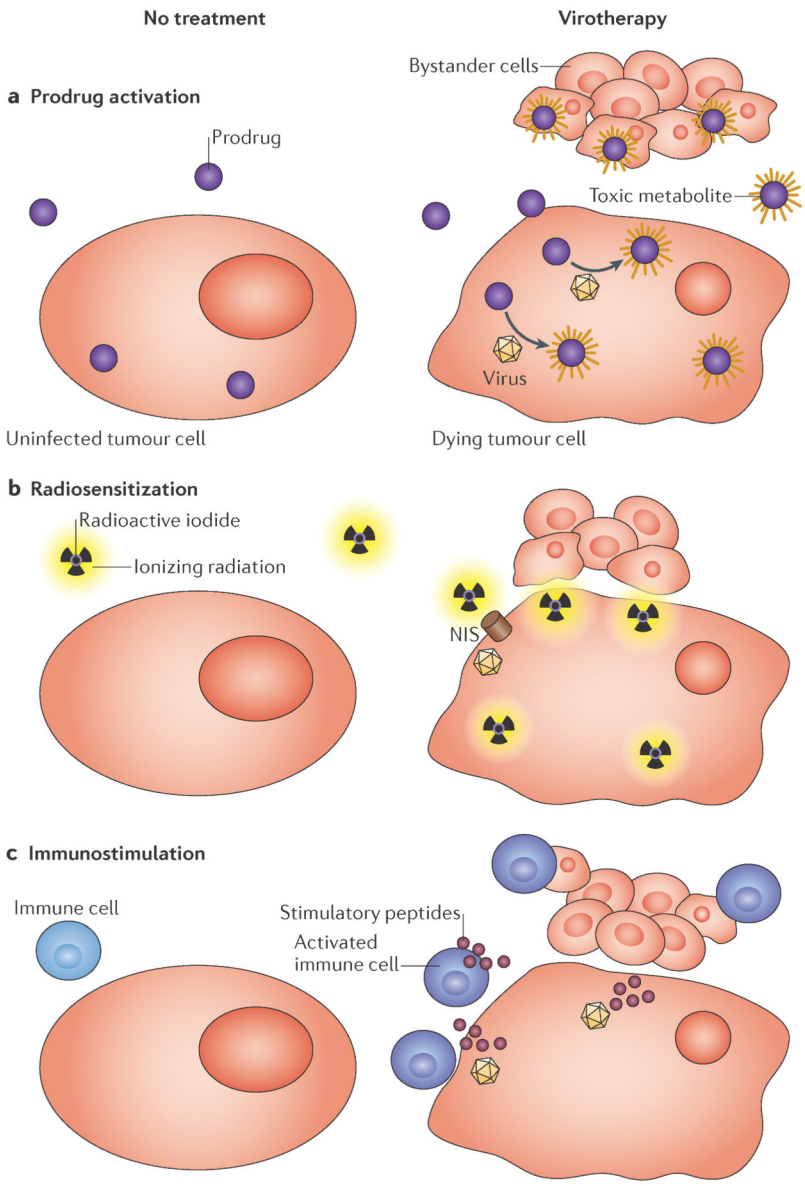
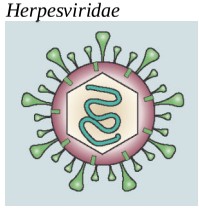
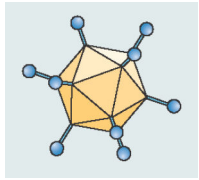
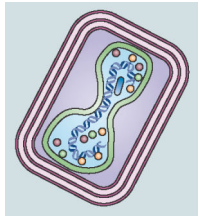


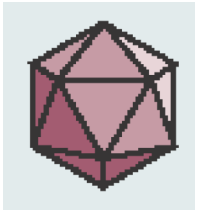
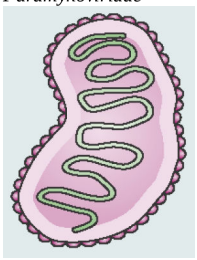
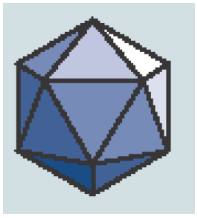
Figure 4. Arming strategies that induce bystander cell killing

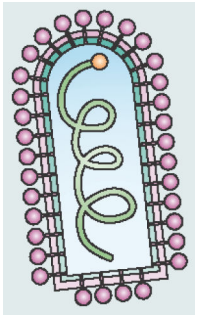
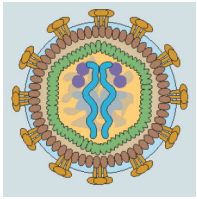
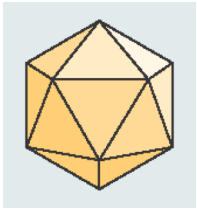
a | Convertase enzymes that are expressed in infected cells metabolize prodrugs into toxic metabolites that diffuse and kill uninfected tumour cells. **b** | The sodium–iodide symporter (NIS) concentrates radioactive ions in infected cells, which induces radiation poisoning of uninfected bystander tumour cells. **c** | Immunostimulatory transgenes that are expressed in infected cells prime responses against tumour antigens, which causes the systemic destruction of tumour cells.

Table 1

Selected examples of current clinical trials with viruses from nine families

Virus family	Phase and type of clinical trial	Virus name	Genetic changes*	Route	Combination therapy	NCT number
DNA viruses						
Herpesviridae 	Phase III complete; melanoma, primary end point met	Talimogene laherparepvec	• Armed with GM-CSF • Tumour-specific replication: ICP34.5 deleted, US11 altered • Enabled antigen presentation: ICP47 deleted	Intratumoural	None	NCT00769704
Adenoviridae 	Phase II and III; bladder cancer	CG0070	• Armed with GM-CSF • Preferential replication in RB-deficient tumours	Intratumoural	None	NCT01438112
	Approved therapeutic (China); SCCHN	Oncorine (H101)	Preferential replication in tumours: E1B-55k deleted	Intravesicular	Cisplatin	PMID15601557
Poxviridae 	Phase IIB; hepatocellular carcinoma	JX-594 (Pexa-Vec)	• Armed with GM-CSF • Preferential replication in tumours: thymidine kinase deleted	Intratumoural	None	NCT01387555; PMID23396206

Virus family	Phase and type of clinical trial	Virus name	Genetic changes*	Route	Combination therapy	NCT number
Parvoviridae 	Phase I and II; glioma	ParvOryx	None	Intravenous; intratumoural	None	NCT01301430
RNA viruses						
Paramyxoviridae 	Phase I; myeloma	MV-NIS	• Armed with human NIS • Preferential replication in tumours (incompetent to block STAT1 and MDA5)	Intravenous	Cyclophosphamide	NCT00450814
Picornaviridae 	Phase I; glioma	PVS-RIPO	Translation controlled by the internal ribosome entry site of another virus	Intratumoural	None	NCT01491893

Virus family	Phase and type of clinical trial	Virus name	Genetic changes*	Route	Combination therapy	NCT number
Rhabdoviridae 	Phase I; hepatocellular carcinoma	VSV-hlFNβ	Armed with human IFNβ	Intratumoural	None	NCT01628640
Retroviridae 	Phase I and II; glioma	Toca 511	Armed with cytosine deaminase	Intratumoural; intravenous; into resection cavity	5-fluorocytosine	NCT01156584; NCT01470794; NCT01985256
Reoviridae 	Phase III; SCCHN	Reolysin	None	Intravenous	Carboplatin paclitaxel	NCT01166542

GM-CSF, granulocyte–macrophage colony-stimulating factor; hlFNβ, human interferon-β; ICP, infected cell protein; MDA5, melanoma differentiation-associated protein 5; MV, measles virus; NCT, national clinical trial; N IS, sodium–iodide symporter; PVS, poliovirusSabin; RB, retinoblastoma protein; RIPO, Rhinovirus–poliovirus hybrid; SCCHN, squamous cell carcinoma of the head and neck; STAT1, signal transducer and activator of transcription 1; US, unique sequence; VSV, vesicular stomatitis virus.

* Changes can be in addition to intrinsic virus properties that favour oncolysis.