Exploiting diversity: Genetic approaches to creating highly potent and efficacious oncolytic viruses
Maxine Bauzon & Terry W Hermiston*

Address
Bayer Healthcare, Pharmaceuticals Division, Novel Technologies Department, 2600 Hilltop Drive, Richmond, CA 94806, USA
E-mail: terry.hermiston@bayer.com

*To whom correspondence should be addressed

Oncolytic viruses possess several key attributes that make them a highly attractive treatment for cancer. They exhibit clinically validated synergy with chemotherapy and an ability to selectively destroy tumor cells to the exclusion of normal cells. Oncolytic viruses can replicate and, therefore, amplify their dose in a tumor-dependent manner. In addition, they can be genetically manipulated to include additional therapeutic factors to create a multimodal anticancer agent. These characteristics lead to the expectation that oncolytic viruses will serve as an additional tool in the treatment repertoire of clinical oncologists. In their clinical development to date, these agents were safe and well tolerated, but lacked efficacy as monotherapies. In this review, three genetic-based methods to increase the potency and efficacy of oncolytic viruses, in which human adenovirus is utilized as an example of a prototype oncolytic virus, are discussed.

Keywords Adenovirus, armed oncolytic virus, cancer, genetic engineering, oncolytic virus, virotherapy

Introduction
Solid tumors are genetically unstable and complex biological systems that challenge both clinicians and researchers. Given this complexity, it is not surprising that current treatments are limited and often unsuccessful at controlling or eradicating human tumors. Moreover, the ability of the cancer cell to adapt to a harsh and ever-changing environment increases the probability that subpopulations of tumor cells will acquire resistance to current therapies. This adaptable nature limits the utility of traditional strategies, such as surgery and radiation therapy. For these reasons, new and more effective cancer treatments are required.

Oncolytic viruses are an appealing cancer treatment class for several reasons. First, by definition, oncolytic viruses can selectively target and destroy tumor cells, while sparing neighboring normal cells. Second, they have the capacity to replicate and, therefore, self-perpetuate in cancer cells. Third, through the genetic incorporation of foreign genes or factors (a process referred to as 'arming'), these agents have the ability to bring together multiple therapeutic strategies into a single agent and attack tumor complexity on multiple levels [1]. Fourth, as oncolytic viruses have the potential to circulate systemically, the treatment of both primary and metastatic lesions is theoretically possible.

Although no longer in its infancy, the field of oncolytic virotherapy has yet to realize its full potential as a novel and effective treatment strategy for cancer [2]. To date, there is only one virus clinically available for cancer therapy, H-101, an oncolytic adenovirus (Ad) that has received regulatory approval in China for the treatment of head and neck cancer in combination with chemotherapy [3,4]. However, with each clinical trial invaluable lessons are learnt that help to direct the field [5••,6]. It has become evident from these trials that oncolytic viruses are safe and well tolerated, but require additional modifications to be efficacious as monotherapies. In this review, three genetic-based strategies for the selection of novel oncolytic agents, with the goal to increase the clinical effectiveness of oncolytic virus therapy, are discussed.

Transductional targeting and detargeting
Tumor cells and their associated endothelial and stromal cells have a variety of cell surface proteins whose expression is critical to the continued growth and development of the tumor (eg, EGF receptor [EGFR], HER-2 [human EGFR 2] and VEGF receptor). These target proteins, their ligands and their associated intracellular pathways can be utilized to discriminate tumor cells from normal cells and have been used in the development of a variety of approved cancer therapies [7]. This improved understanding of tumor biology has been paralleled by an increased knowledge of viral biology, including the identification of viral proteins responsible for attachment and internalization [8], which led researchers to assess the relationship of the cellular receptors required for viral infection and their role or presence in tumor and normal cells. For example, data on the expression of the coxsackie virus and Ad receptor (CAR), the attachment receptor for the clinically approved oncolytic virus H-101, suggest that the lack of abundant CAR expression on a variety of human tumors may represent a significant limitation to the use of this viral agent as a broad and effective anticancer therapy [9]. In contrast,
various viruses under development as oncolytic agents have demonstrated the ability to infect a wide array of both cancerous and normal cells in vitro [10-12]. It is unclear if this lack of tumor-cell specificity will be reflected when viruses are administered to patients with cancer, but this could significantly limit their utility as oncolytic agents. Consequently, a common genetic manipulation to the viral genome is the alteration of a viral protein (or proteins) responsible for the attachment and uptake of the virus into the cell to either gain selectivity (for those that infect cells promiscuously) or to gain efficacy (in cases where the viral agent is extremely lytic [ie, has a high potency for cell destruction], but the native viral receptor required to gain entry is low or absent on the target tumor cell). Strategies to genetically alter viral tropism have been extensively described [13].

One strategy to genetically engineer tumor targeting into the viral attachment or internalization proteins, or both, is to incorporate tumor cell surface-binding proteins identified using phage display technology [14]. While conceptually highly powerful, this strategy has several challenges. It is unclear that the peptides identified in the context of an associated phage coat protein will retain their tumor specificity when placed within the distinctly different oncolytic viral protein responsible for attachment or uptake, or both. In addition, it is unknown how these identified peptides will effect the efficient expression and correct folding of the viral protein. To address these potential deficiencies, 'context-specific' peptide libraries have been developed. For example, a random peptide library is generated between, in this case, the H and I sheets of the Ad fiber protein in the context of the pIII protein of the phage [15]. In this manner, the structural features of the Ad fiber protein and their impact on the peptide selectivity would be represented and could serve to influence the selection of the tumor targeting peptide, thus allowing more faithful replication of tumor selectivity when placed into the full context of the oncolytic viral protein. However, conducting the selection in association with the phage DNA and protein sequences may also incorporate elements that compromise the utility of the sequence for tumor targeting: for example, by impeding or delaying the viral life cycle or by incorporating additional detrimental effects that may only be apparent in a eukaryotic system (eg, changes to splicing signals and glycosylation signals). Research to identify tumor targeting peptides in the context of the Ad fiber protein on the surface of the Ad capsid has been described [16]. Unfortunately, however, these studies were not conducted in a replicating virus and, therefore, do not address all the parameters (eg, efficient attachment, uptake and release of the viral genome to the nucleus and efficient packaging, release and spread of the virus) required for oncolytic viruses. Consequently, a more direct strategy, in which the power of phage library screening to identify tumor-targeting peptides is conducted directly within the context of the oncolytic viral proteins responsible for infection, is required. The realization of this strategy is significantly more challenging, but would allow the identification of viruses that stably incorporate

tumor-specific binding elements and selectively target tumor cells, without compromising the replication efficiency of the virus within the target tumor cells. Approaches to realize this potential are currently under investigation [17].

Exploiting viral serotypes to enhance tumor potency

The majority of oncolytic viruses have been derived from commonly used laboratory strains. For example, all oncolytic Ads to date have been derived from a single serotype, Ad type 5. This preference is primarily a matter of practicality, as this virus and its biology have been extensively studied. Furthermore, Ad 5 is fully sequenced, is readily amenable to genetic manipulation and, importantly, methods for generating high-titer stocks for commercial purposes are well described. However, there are over 50 human Ad serotypes and there is no clear rationale why Ad 5 would be superior to any of the alternative serotypes as a cancer treatment. While some properties of these alternative serotypes have been exploited (eg, exchange of different serotype fiber proteins to alter viral targeting) their rich potential has not been fully explored. This phenomenon of using common laboratory strains is repeated throughout the oncolytic virus field with only rare exceptions [18-20]. Which strain, if any, within a viral class is ideal for the treatment of a specific cancer indication is unclear. Therefore, researchers are challenged to develop new methods to utilize the array of viral serotypes available.

The unique ability of viruses to replicate and spread if a compatible environment is encountered is now exploited to create tumor-directed oncolytic viruses by 'directed evolution' [21*]. In this method, viral strains are combined and passaged in tumor cells that represent the target tumor indication. Passageing of the virus is conducted under conditions that facilitate recombination to additionally increase the diversity and, therefore, the potential to create highly tumor-selective and potent viruses. Through passage and selective replication in the indication-specific tumor cell lines, viruses displaying increased tumor-cell potency are chosen for further study, manipulation and development. In the initial description of this technology, an Ad11p/Ad3 chimeric oncolytic virus, ColoAd1, was generated in colon tumor cells. In vitro, this novel virus demonstrated superior potency and an improved therapeutic window on a panel of colon cancer cell lines and normal cells when compared with the most advanced oncolytic Ad, ONYX-015 (parent serotypes Ad 11p and Ad 3, or Ad 5, the parent for all the current oncolytic Ads). Importantly, these features of increased potency and an improved therapeutic window were reflected ex vivo in surgically derived colon cancer clinical specimens as well as in vivo in a colon cancer liver metastasis xenograft model.

The use of alternative viral serotypes may also address some limitations to current viral therapies. The importance of tumor targeting is equaled by the need to avoid interactions that compromise the viruses' ability to reach the tumor cell. In the case of treatment of metastatic
disease with systemic delivery of the virus, such interactions have great impact. In the case of direct intratumoral injection, liability for interaction is not as great an issue, however, as even neutralizing antibodies do not impact the antitumoral activity [22,23]. For systemic delivery, these interactions involve encounters with components of the bloodstream, including erythrocytes, immune cells and the normal endothelial lining of vessels. For example, it is clear that there is a high prevalence of neutralizing antibodies to Ad5 and that this viral serotype binds tightly to human erythrocytes [24], effects that are currently not reflected in murine tumor xenograft models [25]. Alternative serotypes may, therefore, represent a natural strategy to developing a more efficient systemic therapy; limited interaction with human blood components, both cells and antibodies, may lead to increased delivery of the treatment to all tumor sites and therefore offer the potential for increased efficacy. In support of this hypothesis (or premise), studies using different vaccinia virus strains revealed differences in biodistribution, viral intratumoral spread and immune evasion between strains [26]. Thus, the use of alternative serotypes may offer investigators the most rapid strategy to overcoming some of the limitations that currently challenge the oncolytic virus field.

Arming oncolytic viruses
The ability to express therapeutic genes or factors (eg, short-hairpin (sh)RNA, microRNA or protein fragments) from an oncolytic virus, a process termed ‘arming’, creates a flexibility and complexity not observed with any other existing anticancer treatment [27]. Traditionally targeted at increasing potency, an array of factors has been incorporated that increase the cytotoxicity of the virus by delivering secreted products. These can be broadly grouped into prodru conveying enzymes, immunostimulatory molecules and factors that sensitize the tumor to well-established cancer treatments, such as chemotherapy or radiation therapy [28]. An improved understanding of tumor biology and the requirement to individualize patient treatment has stimulated the development of a new series of factors that could allow the full therapeutic value of oncolytic viruses to be realized. Such factors include: agents that break down the tumor extracellular matrix, which may impede viral spread; agents that allow the investigator to track the virus and its activity in the patient; or agents that could be used to abrogate the viral infection.

A common limitation to various current biological therapies for solid human tumors, including antibodies, is their inability to access and treat the entire tumor mass effectively. The tumor microenvironment can be highly fibrotic, with limited, incomplete and shifting vasculatization marked by areas of hypoxia and low pH; immunoregulatory cell populations can be shifted or neutralized by factors or functions of the tumor cells themselves or their associated stroma. This microenvironment represents a significant challenge that limits many current therapies [29-31]. While the oncolytic virus enjoys the unique ability to replicate and spread upon lysis of the infected cell, viral spread can be limited within the tumor [32,33]. To address this limitation, viruses expressing enzymes (eg, relaxin) to specifically alter the tumor microenvironment have been engineered [34]. The discovery of small regulatory RNAs and the evolution of expression cassettes for their efficient expression has now made these elements a viable complement to the oncolytic virus; for example an shRNA was incorporated into and expressed from a viral genome [35]. The ability to incorporate small regulatory RNAs into the viral genome represents not only an additional factor for arming the oncolytic virus, but also a highly genomically economical method to incorporate therapeutic agents. This genomic economy is important for replicating viruses as they are constrained by the limitations of a viral genome that must be efficiently and stably packaged into a fixed virion coat.

The ability to arm oncolytic viruses also creates a unique opportunity for the clinician to monitor the oncolytic virus therapy during treatment. For example, factors could be incorporated into the virus whose expression and secretion can be easily detected in the bloodstream (eg, serum carcinoembryonic antigen) or whose activity in the presence of selected radionuclides allows for imaging via either SPECT or PET (eg, the human thyroidal sodium iodide symporter and HSV thymidine kinase gene) [36]. If expression of the traceable factor is dependent upon successful initiation of viral replication [37], the clinician would be able to determine the location of the active agent (in the case of SPECT or PET imaging) and the amount of active agent by the level of expression of the secreted, virally encoded traceable factor in the bloodstream or via the imaging signal intensity. The ability to monitor the activity of the viral agent would allow clinicians to move closer to individualized patient treatment. When combination therapies are considered, this information would provide an opportunity to optimize the combination treatment regimen. It is important to note that investigators are not limited to choosing a single therapeutic factor to complement the activity of the oncolytic virus. Efficient multigene expression has been demonstrated for a variety of the viral systems under evaluation as oncolytic viruses [38-40].

In order to maintain the safety of these increasingly potent agents, both the selectivity and specificity of the virus and its therapeutic payload must be enhanced. Initial methods to express therapeutic factors from oncolytic Ads centered on maximizing expression using the human cytomegalovirus enhancer promoter [41,42]. However, the potential to infect non-target cells and express potentially toxic therapeutic transgenes dictated that new, more tumor-specific, expression systems be designed. Consequently, tumor-specific promoters were incorporated as a means to convey specificity to the expression of the therapeutic factor. Unfortunately, many of the oncolytic viruses interact with receptors whose signaling pathway is linked to cell proliferation or tumor formation and metastasis. For example, as a part of their internalization and uptake into susceptible cells, Ad5 interacts with integrins [43] and HSV interacts with integrins, nectin-1
and syndecan-2 [44]. These receptor interactions lead to signaling that is linked to tumor biology [45-47]. Integrins signal through a variety of pathways that include phosphatidylinositol-3-kinase/mitogen-activated protein kinase, Raf1/ERK1/2, and protein kinase C, and these signaling events lead to downstream activation of transcription factors including NFκB. It is therefore plausible that this initial event in viral infection may compromise or ablate the specificity of tumor-selective promoters (especially those responsive to NFκB, such as cyclooxygenase-2, VEGF and survivin [48,49]) and lead to expression in normal cells. Considering the limited packaging capacity of the viral genome and the need to limit therapeutic gene expression to the target tumor cell, endogenous viral promoters are now under investigation. This is a genomically economical strategy as it does not require additional exogenous expression elements to be incorporated into the viral genome. In addition, by utilizing an endogenous viral promoter whose expression only occurs late in viral infection (i.e., after the engineered tumor-selective event), the engineered tumor-selectivity of the virus is linked to expression of the therapeutic factor [28,50**]. In this manner, expression of the armed gene is dependent not simply upon viral entry into a cell, but also upon the specificity of the oncolytic viral infection, thereby linking selectivity of the viral agent to expression of the therapeutic factor. If DNA replication is absent because of lack of infection or aborted infection of non-target cells, no therapeutic factors should be expressed. However, the therapeutic factor will be expressed in a target cell that supports the full viral lifecycle. Viral expression systems based on this principle have now been developed [37,51,52]. Smaller splice acceptor sequences can also be utilized to control the expression of therapeutic factors from an endogenous promoter in a DNA replication dependent manner [37].

As mentioned previously, alternative viral strains may hold great potential for the development of novel and improved oncolytic agents. It is important to recognize that the biology or sequences of these alternative viral strains are unlikely to be well defined. Consequently, systems that can scan viral genomes for sites compatible with therapeutic factor insertion that would not compromise the viral life cycle are required. An initial system, based on a modified Tn7 transposon system, was used to identify unique insertion sites within a replicating human Ad genome [53]. Interestingly, changes in the expression cassette (e.g., altering the promoter contained within the expression cassette from strong to weak or modifying the splice acceptor sequence so that it was more like or less like the consensus splice acceptor sequence) altered the sites of transposon insertion [37,53]. Consequently, this strategy creates an opportunity to identify sites that are compatible with the viral life cycle and maximal expression of the therapeutic factor, regardless of the expression cassette used. Importantly, this system is not dependent upon previous knowledge of the biology or sequence of the virus.

Conclusion

Oncolytic viruses represent promising cancer treatments, but, unfortunately, they have yet to realize their full potential. As with all therapeutics, it has been important to establish the safety of these agents. However, despite promising safety profiles, oncolytic viruses have had limited success as monotherapies. The limitations of early oncolytic viruses have been recognized and are being addressed by several strategies in an ongoing attempt to increase the efficacy of these agents; this review has focused on three genetic strategies. Our understanding of tumor and viral biology has allowed an improved understanding of the challenges associated with some of the current oncolytic agents, specifically at the level of attachment and uptake. Significant research is ongoing and new novel strategies are being utilized to address this need. For example, the use of alternative strains, either from nature or developed in the laboratory, is providing starting materials with improved infectivity, potency, and systemic potential. In addition, the expression of selected exogenous genes has been demonstrated to increase intratumoral spread. An understanding of these agents in the clinical setting is expected to be enhanced by the ability to track and monitor the oncolytic agent. Arming continues to hold significant promise for providing a method of delivering additional therapeutic agents to the tumor cells, as well as the flexibility required to overcome the hurdles facing these virotherapies (e.g., the inability to access the tumor and limited spread throughout the mass). The strategies outlined in this review to improve viral efficacy are complemented by research to optimize combination therapies that include current standard of care chemotherapy, radiation, and biologicals (e.g., antibodies). Additional research (e.g., administration of clinically approved immune suppressor molecules such as cyclo-phosphamide) is tailored specifically to the oncolytic virus and its unique potential requirements as a therapeutic. The hurdles to developing a successful cancer therapeutic are not unique to oncolytic viruses. On the contrary, the challenge exists for all established and developing cancer treatments. However, the tremendous potential and unique properties that replicating viruses hold for the treatment of cancer make for an exciting and dynamic time as these agents move toward clinical approval.

References


• This study highlights the potential for species-specific interactions between the virotherapy and blood components.


