Reovirus: A Targeted Therapeutic—Progress And Potential

Radhashree Maitra, Mohammad H. Ghalib, and Sanjay Goel

Abstract

Medical therapy of patients with malignancy requires a paradigm shift through development of new drugs with a good safety record and novel mechanisms of activity. While there is no dearth of such molecules, one particular agent, “reovirus” is promising by its ability to target cancer cells with aberrant signaling pathways. This double-stranded RNA virus has been therapeutically formulated and has rapidly progressed from preclinical validation of anticancer activity to a phase III registration study in platinum refractory metastatic squamous cell carcinoma of the head and neck. During this process, reovirus has shown safety both as a single agent when administered intratumorally and intravenously, as well as in combination therapy, with multiple chemotherapeutics such as gemcitabine, carboplatin/paclitaxel, and docetaxel; and similarly with radiation. The scientific rationale for its development as an anticancer agent stems from the fact that it preferentially replicates in and induces lyses of cells with an activated Kras pathway. As documented in many previous studies, the initial observation of greater tropism in Kras-compromised situation might certainly not be the sole and possibly not even the predominant reason for enhanced virulence. All the same, scientists have emphasized on Kras optimistically due to its high prevalence in various types of cancers. Incidence of Kras mutation has been found to be highest in pancreatic cancer (85–90%) followed by colorectal (35–45%) and lung (25–30%). Reovirus, in fact has the potential not only as a therapy but also as a tool to unravel the aberrant cellular pathway leading to carcinogenicity. Mol Cancer Res; 1–12. ©2012 AACR.

Introduction

Exciting research in the past decade has revealed unique viral characteristics not registered in any other microorganism. Viruses harbor distinct strategies to overcome the sophisticated defense mechanisms of the infected host (1). The intricate ability to quickly gain control over the host cellular mechanism has potentially made the virus a double-edged sword. While several viruses have been identified as cancer-causing agents, many have also been identified as therapeutically viable oncolytic elements. One important member of therapeutically identified viruses is Respiratory Enteric Orphan virus commonly known as REOvirus (1). It is naturally oncolytic with inherent propensity to replicate in cells with dysfunctional cell signaling cascade including Ras-activation. Preferential oncolytic properties of reovirus can be effectively exploited as clinical therapy due to general mildness of infection often requiring no special medical intervention.

The search for oncolytic viruses roots from the fact that transient cancer remission can occur following viral infections (2). Supportive preclinical evidence of the novel mechanisms of anticancer activity of reovirus provided the rationale for therapeutic advancement to clinical trials. Unique mode of tumor destruction encouraged oncolytic viral therapy as potential augmentation of chemotherapy and radiation thus making it a cutting edge clinical approach (3, 4).

Many of the oncolytic viruses currently in clinical testing are attenuated derivatives of prevalent human pathogens. Typically, in recent years, they have been genetically engineered to further reduce their pathogenicity and increase their oncolytic potency and enhance specificity for cancer tissue. Virus with a double-stranded DNA genome is the most suitable candidate for such manipulations with greater genome stability and lesser chance of hazardous mutations. Adenoviruses and herpes simplex virus are the most suitable and thus been extensively engineered. Reverse genetic manipulation of RNA virus is still a scientific challenge even with the availability of the present day cutting edge genetic technology. In a similar vein, reovirus is not amenable to genetic engineering, especially due to its segmented structure of the dsRNA (5). However, considering the fact that this naturally oncolytic, and pathologically self resolving virus seems to be tumor cytotoxic, even in the presence of neutralizing antibodies, the lack of engineerability may not be a constraint to its further therapeutic development.
Preclinical Studies with Reovirus

Reovirus as a single agent

In vitro data with reovirus. Several preclinical studies documented oncolytic characteristics of reovirus (6, 7). Initial studies with NIH-3T3 cells revealed that resistance to reovirus infectivity can be overcome by transformation with activated Ras oncogenes (8, 9). The findings indicated that usurpation of Ras signaling pathway constitutes the basis of viral oncolysis (8). Several in vitro studies with human cancer cell lines documented the evident role of reovirus in cellular cytopathy (4, 8). A systematic analysis of the propensity of reovirus infectivity towards 24 established glioma cell lines was conducted. Dramatic and widespread cell killing after exposure to live (but not dead) reovirus occurred in 20 (83%) cell lines. After 48 hours of infection, widespread cell death was found in U87, U251N, and A172 cell lines, and almost complete cell death was seen after 72 hours. In contrast, cells receiving either dead or no virus remained healthy. Furthermore, to ensure that cell lysis was due to viral replication, cells were reacted with rabbit anti-reovirus antibody, followed by FITC-conjugated goat anti-rabbit IgG when susceptible lines depicted the expression of the viral antigens. Replication of reovirus in susceptible lines was further confirmed by [35S]methionine metabolic labeling (4).

Short-term primary culture from surgically excised human glioma have shown 100% sensitivity to reoviral oncolysis (4). Studies conducted to test the ability of reovirus to infect and kill primary cultures of brain tumors freshly excised from 9 patients with glioma intriguingly depicted complete growth arrest and cell death (100%), although similar level of reovirus-mediated cellular cytopathy was not documented with 7 primary meningioma cultures (4). The ability of reovirus to lyse all primary glioma cell cultures derived from surgical specimen suggested that a substantial proportion of gliomas may respond to reovirus treatment. It can be argued that the number of specimens being relatively small, the observations might not reflect the comprehensive tumor properties of gliomas. The oncolytic spectrum of reovirus has also been studied in 6 breast cancer cell lines where a high susceptibility was documented (10). An infectivity of 10 MOI (multiplicity of infection) was used in the study. Furthermore, the control Hs578Bst normal mammary gland epithelial cell line used in the study did not show any cytopathic effect (CPE) to the virus confirming the fact that reovirus preferentially targets the transformed cells sparing the normal ones (9). Contemporary studies with melanoma cell lines and primary cultures from fresh resected tumors revealed similar results. The transformed cells allowed viral replication followed by caspase-dependent cytotoxicity (11). Normal melanocytes conversely resisted viral replication. Notably, it was also reported that all of the screened breast cancer and melanoma cell lines had activated Ras.

The basis of the ability of reovirus to target and kill tumor cells but not infect nonproliferating normal cells lies in its ability to usurp the highly activated signaling pathway found in tumor cells (4). This ability is most clearly established for Ras or elements in its downstream pathways. Ras activation is very common in malignant gliomas, colorectal (CRC) cancers, as well as pancreatic malignancies.

Natural affinity of reovirus to kill and lyse cancer cells and the prevalence of Kras mutation in many of the studied models apparently instigated the researchers to attempt to draw a correlation between Kras mutation and viral oncolysis. It is to be noted that the fact is yet to be substantiated with scientific evidence. A somewhat similar situation was faced by the scientific community while developing the therapeutically viable adenovirus Onyx-01. The antitumor activity of the virus was initially proposed to be solely dependent on the status of p53 but deeper introspection revealed that lytic activity was observed in both p53 negative and WT conditions and the complex molecular mechanism of virus mediated oncolysis is yet to be clearly defined (12).

Further studies to elucidate cellular events favoring reovirus-mediated apoptosis confirmed that colon cancer cell lines, HKE293 and HCT116 displayed elevated β-catenin expression to promote reovirus-mediated oncolysis by down-regulation of NF-κB (3). Independent studies reported that reovirus activates human dendritic cells to promote innate antitumor immunity (13). The exact role of different immune effector cells in oncolytic virus-mediated tumor regression has not yet been clearly defined. It is plausible that dendritic cells (DC) are likely to play a coordinating role in virus-mediated immune response as key antigen presenting cells (APC) that recognize the viral infection and regulate both innate and adaptive immunity (11). The fact that reovirus has been found to be effective in tumor cytopathy despite increasing neutralizing anti reovirus antibody (NARA) in the serum logically indicates the intricate role of the innate immune system in the virus-mediated oncolytic process. The observation that reovirus-activated DCs enhance the innate natural killer (NK) cells and cytotoxic T cells (Tc) by release of soluble factors inducing tumor cell killing via exocytosis clearly supports the hypothesis. The role of NK cells in tumor regression has been well documented in mouse model both by direct tumor recognition as well as via DC activation (14). Reovirus-induced DC maturation also stimulated the production of proinflammatory cytokines interferon (INF)-α, tumor necrosis factor (TNF)-α, interleukin (IL)-12p70, and IL-6. Activation of DCs by reovirus was not dependent on viral replication, whereas cytokine production was inhibited by the blockade of PKR (protein kinase receptor) and NF-κB signaling. These observations provide a hint that reovirus-mediated DC activation and the downstream immune signaling is multimodal. Although systemic delivery activates the adaptive immune system and triggers a robust antibody response, intratumoral (ITu) injection of the virus results in successful tumor destruction by activation of the innate immune effector cells within the tumor microenvironment. Hence, reovirus recognition by DCs may trigger innate effector mechanisms to complement the virus’s direct cytotoxicity, potentially enhancing the efficacy of reovirus as a therapeutic agent (13). Intravenous administration of reovirus in conjunction with immunosuppressants has been found to be therapeutically more effective indicating that the neutralizing antibodies are not completely blunted (15).
the fact that antitumor activity is observed even in the presence of virus-neutralizing antibodies can also indicate a plausible role of the host cellular machinery in camouflaging the virus and thus preventing its recognition by the specific antibodies. In a very elegantly conducted translational research study, it has been shown that replicating virus is detectable in cellular compartment of the blood, namely in mononuclear, granulocyte and platelets, and this may be a mechanism of protection from NARA (16). Furthermore, in this same clinical trial, when patients’ tumors were harvested, replicating virus was evident in tumor tissue but not from normal liver, showing some evidence of selective tropism for malignant cells.

PKR plays crucial inhibitory role in efficient viral replication essential for infective virion production and oncolysis (refs. 7, 17; Fig. 1). Expression of PKR is upregulated in response to INF released by infected cells (7). Binding to viral RNA/initial transcripts results in PKR dimerization, autophosphorylation, and activation (7). The viral S1 segment mRNA has been shown to be a potent activator of PKR (18). Once activated, PKR blocks the primary and secondary reovirus protein translation. In Ras-transformed cells, PKR is not activated, allowing unabated viral replication and effective assembly of viral proteins for production of infection efficient virions (7, 17). Specific chemical inhibitors of PKR phosphorylation restore reovirus translation in untransformed cells providing evidence for a direct role of PKR in defining resistance to reovirus replication (9). Ras activation is suggested to release the translational blocks in the transformed cells. However, the exact molecular mechanism of coordination between Ras activation and inhibition of PKR-mediated viral translational remains elusive (Fig. 1).

The recently discovered novel oncogene CUG2 (cancer upregulatory gene-2), inhibits the expression of PKR and activates Ras and p38 MAPK (19). Studies further confirmed that inhibition of p38 MAPK or Ras blocks reoviral proliferation even in the presence of CUG2 indicating the possibility of multimodal crosstalk between Ras activation and inhibition of PKR phosphorylation (19). It is pertinent to mention that PKR dimerization and autophosphorylation is critical for effective viral propagation but the definitive contribution of Ras mutation in the process is still elusive. Aberrant cell signaling cascade generates many anomalous events within the cell and exactly how it facilitates the inhibition of PKR dimerization is yet to be defined clearly. Investigation of the mechanism of apoptosis in reovirus-infected HEK293 cell line concluded a significant role of TRAIL (TNF-related apoptosis-inducing ligand). The apoptotic event was successfully inhibited by anti-TRAIL antibodies or death receptor (DR)4 and DR5 (20). Similar involvement of TRAIL was confirmed in cell lines derived from 2 different human lung (A157/H549) and breast cancers (MDA231/ZR75-1; refs. 20, 21). Reovirus infection synergistically sensitizes these cancer cell lines to killing by exogenous TRAIL. The observed sensitization was associated with an increase in the activity of the death receptor-associated initiator caspase-8, and was inhibited by the peptide IETD-fmk, suggesting that reovirus sensitizes cancer cells to TRAIL-induced apoptosis in a caspase-8–dependent manner. Enhanced sensitization was also found to be associated with increased cleavage of PARP, a substrate of the effector caspases 3 and 7 (22).

In vivo experience using animal models of cancer. Tumor regression studies in animals with reovirus as single agent showed dramatic results. Pronounced effects often with complete tumor regression was documented in vivo in 2 subcutaneous (P = 0.0002 for both U251N and U87) and in 2 intracerebral (P = 0.0004 for U251N and P = 0.0009 for U87) human malignant glioma mouse models (4). Immunocompetent C3 mice implanted with Ras-transformed C3H10t1/2 fibroblast showed complete regression of tumor after ITu injection of reovirus (23). Similar strategy of ITu injection resulted in significant tumor regression in SCID mice with subcutaneously implanted v-erbB–transformed NIH-3T3 cells (23), and in SCID-NOD mice with subcutaneously implanted human malignant glioma (4, 8). Subcutaneous tumor allograft studies in immune competent C3H mice showed strong inhibition of tumor growth with intravenous administration of reovirus (15). A 74% cure rate was observed in comparatively less immunocompromised nude mice harboring human malignant glioma with single intraleus injection of reovirus. SCID-NOD mice bearing subcutaneous U251N xenografts (4) or multiple myeloma RPMI8226GFP+ cells introduced via tail vein for tumor establishment, when treated with single injection of live or dead reovirus showed a striking regression of live virus–treated tumors (24). Immunofluorescence analysis showed that reovirus replication was restricted to the tumor mass without spreading to the underlying normal tissue.

Reovirus as combination therapy

Association with radiotherapy. Combination therapy involving radiation and reovirus was evaluated both in vitro and in vivo (21). The CRC cell line HCT116 was treated with reovirus and radiation individually, and in combination, showed a marked synergy in the combinational subset. In vivo murine studies depicted similar synergy in nude mice with tumor induced by HCT116/SW480 cell implantation as well as in C57BL6 mice by B16 melanoma cells mediated tumor induction (21).

The relative tumor volume (RTV) when measured in nude mice xenografted with RH30 and SK-ESI human sarcoma and treated with similar doses revealed lowest mean tumor volume and longest event-free survival in the group receiving the combination treatment. TUNEL assay conducted with tumor biopsies showed enhanced apoptotic activity in combination treatment. TUNEL assay conducted with tumor biopsies showed enhanced apoptotic activity in combination therapy as compared with single agent (25).

Association with chemotherapy. In vitro studies to determine augmentation of viral therapy with chemotherapy was done using gemcitabine, fluorouracil, cisplatin, and doxorubicin in HCT116 cells. Reovirus was synergistic with all 4 drugs across a wide range of concentration (26). The synergistic consequences observed with gemcitabine advanced further onto in vivo evaluation of xenografted nude mice. A remarkable synergy was confirmed with no
residual tumor tissues remaining at the end point of the study in more than 95% of the mice (27).

Another independent study of reovirus in combination with cisplatin, mytomycin, vinblastine, or gemcitabine in NSCLC cells lines documented synergy in cell lines sensitive to the chemotherapeutic drugs and antagonism in cell lines that are drug resistant. The taxanes showed reasonable synergy when administered in combination even in drug-resistant cell lines (28). Similarly C57BL/6 rodent with B16 melanoma–induced tumor when treated with combination therapy of reovirus and cisplatin showed a synergistic reduction in RTV as compared with those receiving monotherapy (29).

In context of animal models of human tumors, athymic mice bearing xenografts of osteosarcoma, Ewings, and synovial sarcoma when treated in similar fashion also confirmed a synergistic antitumor activity in comparison with monotherapy (30). Docetaxel administered in combination in cell lines and PC-3 prostate cancer mouse model confirmed synergy, which diminished with increasing docetaxel concentration (31). The confinement of viral proteins within the tumor mass was confirmed by several studies including

![Figure 1. A schematic illustration of the current hypothesis of the fate of reovirus upon entering the host cell by phagocytosis. The status of the host cell signaling cascade determines the downstream consequences. Kras (Kirsten rat sarcoma viral Oncogene) is a membrane-bound molecular switch which binds to and catalyses the hydrolysis of GTP (guanosine triphosphate) to initiate a cell signal. The hydrolysis is facilitated by GTPase activating protein (GAP) and guanine nucleotide exchange factor (GEF) with subsequent conversion to inactive Kras GDP (guanosine diphosphate) complex. On the left is represented the k-ras wild-type (WT) environment, which prevents the translation of viral transcripts and abrogates reovirus assembly. The double-stranded RNA (dsRNA)-dependant protein kinase R (PKR) plays a crucial role in the viral translation block by dimerization and autophosphorylation to phospho-PKR (P-PKR), which in turn promotes the phosphorylation of translation initiation factor 2 subunit alpha (eIF2α) to P-eIF2α. The dashed arrow depicts the translational inhibition by P-eIF2α in Kras WT cells. In Kras–compromised situation (shown on right), the autophosphorylation of P-PKR is inhibited with unabated viral translation and subsequent virion assembly and cell lysis. The factors contributing to the crosstalk between defective Kras and compromised PKR phosphorylation are unknown and thus represented by (?). Cancer upregulatory gene 2 (CUG2) frequently elevated in variety of tumor tissues has been recently characterized to promote reoviral proliferation by activation of Kras and inhibition of PKR phosphorylation. CUG2 has been proposed as a biomarker to identify the subset of cancers that might benefit from reoviral therapy.]

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immunohistochemical evaluation of U87 xenografted rodent (8). The broad oncolytic spectrum of reovirus as single agent and in combination along with remarkable success of the animal study platform provided the scientific confidence to proceed onto clinical trials. Although much preclinical work has been carried out establishing that reovirus could serve as a cancer therapy, the mechanisms governing the permissiveness of transformed cells to reovirus infection remain to be fully characterized. Furthermore, as reovirus replicates in cancers of very diverse origin, it is likely that the virus exploits cellular signals that occur often in transformation and tumorigenesis. A deeper understanding of the molecules involved in antiviral defense and the patterns they recognize will allow harnessing them for better therapeutic strategies. Thus, delineating this usurpation should in turn shed light on signaling that is common among various cancers, and may reveal novel therapeutic targets.

Although the first hint of reovirus susceptibility (32) was noted in 1977, it was not until 1990s that any scientific clue was obtained regarding the preferential replication of reovirus in transformed cells. Since then, systematic research has provided some clues on the molecular synergy of efficient reovirus oncolysis in *ras*-compromised cancer cells. Although the preliminary finding might not be substantial in defining the viral tropism, it has nevertheless logically permitted clinicians to attempt the use of reovirus as treatment towards *ras*-mutated carcinogenicity especially in situations where no other well established treatment regime is available.

Incidentally, reovirus is intrinsically oncolytic without need for any genetic manipulation. It is a naturally occurring virus that possesses the right level of potency that renders it relatively harmless to normal cells while having strong lytic effect on *ras*-compromised cancer cells. Investigations on the mechanisms of selective viral oncolysis in *ras*-mutated tumor cells will help unravel the complex cellular pathways involved in cellular transformation which in turn will not only help in identifying novel targets for cancer therapy but also shed light on which cancer backgrounds are compatible with viral oncolysis strategies.

**Clinical Experience with Reovirus**

Reovirus has been therapeutically tested in 325 (as reported) patients administered ITu or intravenously either as a monotherapy or in combination with radiotherapy or chemotherapy. The first human trial with reovirus began in July 2001, and since then a total of 27 clinical trials have been completed/initiated/deplored as phase 1, 2 and 3. Thirteen trials have been completed, whereas 12 are ongoing and 2 have been announced. Of these 27 trials, 6 will be sponsored by the National Cancer institute. Of the 16 trials with reported clinical data, clinically formulated reovirus was injected ITu in 6 trials enrolling 90 patients, whereas the remaining 10 clinical trials involved the intravenous route enrolling 235 patients (Table 1). The current dosing regimen is intravenous of 3 × 10^{10} TCID_{50} on days 1 to 5 over 60 minutes repeated every 3 or 4 weeks in both mono- and combination therapy. As with the preclinical observations, the clinical experience with reovirus has been interesting, evolving, and has shown promise. Summaries of the results are provided in Table 1.

**Reovirus as intratumoral injections**

The first human phase 1 trial was conducted in 18 patients with metastatic or recurrent solid tumors with easy access to the tumor to allow for direct ITu injection, and measurement by direct observation or palpation. The treatment was well tolerated without any observation of dose-limiting toxicity (DLT) and/or maximum tolerated dose (MTD; ref. 33). The second trial was based on the preclinical experience of reovirus on prostate-derived cell lines (34). Six patients with prostate cancer (stage T2/T3) received a single transrectal ultrasonography (TRUS)-guided reovirus injection followed by radical prostatectomy after 3 weeks. Histologic analysis after prostatectomy of the injected lesions and other synchronous lesions showed CD8 T-cell infiltration and evidence of caspase-3 activity within the reovirus-injected areas. The third clinical trial was based on the preclinical data showing reovirus activity in malignant gliomas (4). While oncolytic viruses have been used in clinical trials in malignant gliomas (35–38), this study was the first to use reovirus. A total of 12 patients (10 glioblastoma multiforme, 1 anaplastic astrocytoma, and 1 anaplastic oligoastrocytoma) were treated at 3 dose-escalating levels (ongoing trial). Antibody studies showed a seroconversion rate of 83% consistent with other clinical studies. Subsequently, a combined phase 1/2 study (currently ongoing) was started in recurrent malignant gliomas with a single ITu infusion (using transcranial catheters) of reovirus directly into the intracerebral tumor over 72 hours. Accrual to the phase I portion has been completed, but the data are pending.

In another 2-stage phase I trial, dose-escalating reovirus was given ITu along with palliative radiotherapy to 23 patients with advanced cancers (39). There was no evidence of exacerbation of skin toxicity induced by radiation. There was marked efficacy with 7 of 14 evaluable patients having experienced a partial response (PR). In another phase 2, multicenter, clinical trial, ITu reovirus was evaluated when combined with low-dose radiotherapy (20 Gy) in patients with advanced cancer (40). This study showed safety and efficacy of the combination with 4 patients with PR and 2 with minimal response.

**Reovirus as systemic administration**

There have been 2 single agent, phase 1 trials of reovirus as a systemic intravenous infusion. One clinical trial was conducted at our center, Montefiore Medical Center, Albert Einstein College of Medicine (Bronx, NY; ref. 41). This was a single center dose-escalation trial and enrolled 18 patients with refractory solid tumors. Overall, toxicities were minor, with only 2 patients experiencing grade 2 events, without an observation of a single DLT. One patient with breast cancer who received 7 cycles developed grade 2 fever and grade 1 chills that progressively worsened with repeated
Table 1. Summary of clinical trials with reported data. This table summarizes the current data from completed and ongoing clinical trials with Reovirus.

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<th>Study #</th>
<th>Indication</th>
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<th>Number of patients</th>
<th>Phase</th>
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<th>Highest dose (TCID$_{50}$)</th>
<th>Grade 3/4 toxicities</th>
<th>Efficacy</th>
<th>Viral detection serum/CSF/stool/urine/feces</th>
<th>Antibody response</th>
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<td>$3 \times 10^{10}$</td>
<td>June 26, 2017. © 2012 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from 960 MC.12-0157 1/6</td>
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<td>NR</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>NSCLC</td>
<td>Carboplatin AUC 6 Paclitaxel 200 mg/m\textsuperscript{2}</td>
<td>22</td>
<td>2</td>
<td>$3 \times 10^{10}$</td>
<td></td>
<td>Myelosuppression Hypokalemia Fatigue Anemia Nausea Hypotension</td>
<td>6 PR, 13 SD</td>
<td>NR</td>
<td>NR</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Pancreatic cancer</td>
<td>Gemcitabine 800 mg/m\textsuperscript{2}</td>
<td>18</td>
<td>2</td>
<td>$3 \times 10^{10}$</td>
<td></td>
<td>Neutropenia</td>
<td>1 unconfirmed NR PR</td>
<td>7 SD</td>
<td>NR</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** The study # preceded by "REO" indicates the number assigned by the study sponsor, namely Oncolytics, to enable an easy search for the reader. While some trials are mature and full reports have been published, others are ongoing with preliminary data presented at international and national meetings or updated on the company’s website. The table provides a quick overview with focus on the study design, patient accrual numbers, dosing, immunology, toxicities, and efficacy, and comments about the uniqueness of the conduct or the results of the study.

Abbreviations: AUC: area under the concentration time curve; XRT: radiation; MR: minimal response; NR: not reported; PFU: particle forming units.
administration. She had a PR and her tumor had a mutation in ras codon 12 (42, 43). In the second, 33 patients enrolled, of which 10 patients showed stable disease (SD; ref. 44). Overall, 28 patients showed an increase in NARA titers to a maximum at 4 weeks, which remained constant during subsequent cycles (45). Despite the NARA response, which could have blunted the viral delivery to tumor site (via rapid neutralization), viable virus was shown in post treatment biopsies after 2 cycles (45).

More recently, in a phase 2 study that enrolled patients with advanced CRC with liver metastasis, reovirus was given intravenously for 5 consecutive weeks before the planned liver metastectomy. Among the 10 patients treated so far, there is evidence that reovirus selectively targets tumor cells versus normal liver cells (60% of patients have shown no evidence of reovirus in their normal liver cells). In 2 patients, only necrotic tissue was seen, whereas reovirus was detected in the immune cells of the tumor of 1 patient. This new exciting information shows that reovirus can be delivered specifically and selectively as a monotherapy while sparing the normal liver cells (ongoing trial). The first completed phase II trial of single agent reovirus targeted 53 patients with soft tissue and bone sarcomas with metastasis to lung. A total of 19 patients showed SD. One patient with synovial sarcoma had SD for more than 80 weeks.

There is strong preclinical rationale to combine intravenous infusion of reovirus with cytotoxic chemotherapy like gemcitabine, carboplatin, or docetaxel (15, 27, 28, 46, 47). The phase 2 dose of reovirus was based on two-phase 1 monotherapy trials (41, 44). It was postulated that chemotherapy might blunt the immune-mediated viral clearance without significantly increasing the toxicity profile. In the first combination phase I study, intravenous reovirus (on day 1–5 of each cycle with the starting dose at 3 \times 10^{10} TCID_{50} \text{ day}^{-1} \text{infusion}) was administered with gemcitabine at 1,000 mg/m² on day 1 and day 8 (21-day cycle) in dose-escalating cohorts (48). However, with the observation of 2 DLTs [grade 3 ALT (alanine aminotransferase) rise and grade 3 Troponin I] the dose of reovirus was amended to a 1-day treatment. One patient with nasopharyngeal carcinoma [(NPC)] which is caused by an Epstein Barr virus (EBV) infection] showed a PR, possibly due to the expression of EGFR (EBV-induced expression). The combination showed clinical activity despite the potential NARA response, and ongoing work is unraveling the intricate details of mechanisms involved in reovirus-mediated oncolysis and resistance (49, 50). On the basis of this clinical (48) and prior preclinical findings (51), a phase 2 clinical trial in patients with metastatic pancreatic cancer is ongoing, and preliminary data suggests a clinical benefit rate of 58% with prolonged SD (52). Preclinical data suggestive of hepatic toxicity associated with reovirus (53) and the observation of the ALT elevation when combined with gemcitabine (48) should be factored in dealing with situations where patients have compromised hepatic function or are taking potentially hepatotoxic medications, such as acetaminophen.

Therapeutically formulated reovirus has been combined with docetaxel in 24 patients with advanced cancer (54). Of all patients treated, 46% experienced grade 3 or greater neutropenia, consistent with that observed with docetaxel monotherapy (65%) at the same dose and schedule. MTD was not technically reached as only one DLT, of grade 4 neutropenia, was encountered. Four PR were observed in breast, stomach, goserosophageal, and ocular melanoma along with 3 minor responses in mesothelioma, prostate cancer, and squamous cell cancer of the head and neck. Only 2 patients showed evidence of viral shedding. Pharmacokinetic studies revealed no change in docetaxel clearance by the addition of reovirus. Docetaxel showed no effect on NARA, a finding that was inconsistent with preclinical data (55).

In a separate phase I trial, reovirus was added to the combination of carboplatin and paclitaxel (56). With preliminary signs of clinical activity, the phase 2 expansion cohort was enriched with patients with squamous cell carcinomas of head and neck (HNSSC), and remarkably, 8 of 19 (42%) evaluable patients showed PR. The toxicities were consistent with earlier results. This study showed the safety and efficacy of this combination, leading to a targeted phase II study in patients with HNSSC with a RR of 31% (57), and finally to a randomized phase III study of carboplatin and paclitaxel with or without reovirus in patients with platinum refractory HNSSC (ongoing trial). The same combination has also been tested in patients with advanced non–small cell lung cancer (58). As reported at the 14th World Lung Cancer Congress, the combination was well tolerated and of the 23 patients entered, 6 patients had a PR and 13 had SD.

Neutralizing anti-reovirus antibodies

An important and critical issue when considering the clinical use of reovirus is the presence of preexisting and the development of a rapid massive rise of titer of “on therapy” NARA. This phenomenon of NARA development has been consistently observed across the single agent and in the chemotherapy combination trials. It is clearly evident that despite the presence of preexisting NARA, there is a detectable 100-fold increase in the viral titer, which has been suggested to be both desirable and unwarranted. On one hand, it allows for limiting the toxic effects of the virus and protects patients from the unwanted side effects of the virus infection, whereas, on the other, it may compromise its beneficial potent anticancer effects. Prior preclinical in vivo studies in murine models using C3H mice have shown that blunting the immune response using cyclosporine A or anti CD4/CD8 antibodies leads to improved efficacy (15). Similarly, cyclophosphamide, when used as a modulating agent, was effective in ensuring tumor seeding of the intravenously delivered reovirus in tumors, an entity which was previously not attainable (59). By modifying the dose of the cyclophosphamide, the authors also showed that it modulates, but does not ablate, the NARA response.

The concomitant delivery of reovirus with chemotherapy has been purported to be of benefit, partly by the attenuation of the NARA response. However, in combination chemotherapy trials, specifically with docetaxel, and carboplatin–paclitaxel, there was a significant rise in NARA titer

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and no discernable increase in toxicity (46, 56). Another interesting phenomenon observed in these trials was that in comparison with the single-agent studies, the rate of rise of the NARA titer was lower, thereby leading to a delay in the achievement of the peak titer. Slower development of NARA may have beneficial effects by enhancing tumor seeding of the virus leading to greater efficacy without compromising safety. Interestingly, the only agent that had some suggestion of the ability to blunt the NA response was gemcitabine (48). This is not surprising as gemcitabine has been shown in murine models to specifically affect the generation of antibodies by B cells (49) and to suppress myeloid suppressor cells (60). Furthermore, independent of the attenuation of the NARA response, gemcitabine has been reported to tip cellular immunity favoring reovirus-initiated antitumor immune response (51, 60). The attenuated NARA response allowed for greater virus replication leading to greater toxicity of the combination as compared with the single-agent profile, an important but not entirely unexpected clinical observation. Therefore, careful monitoring of immune function should remain an important consideration in future combination trials. Moreover, it has been suggested that because of the interference of the adaptive immunity of the host, the most effective systemic delivery of reovirus will be achieved through rapid, repeated high doses of virus within the first week of treatment before the NARA response has been boosted (45). The current dosing schema of 5 continuous daily intravenous injections of the virus every 3 to 4 weeks, therefore, has some clinical and scientific rationale.

Summary, Conclusions, and the Future

In summary, reovirus has shown promise with far reaching implications for future drug development. While initially developed as an anticancer agent based on the scientific premise that viral replication is supported in ras-driven cancer cells; the paradox lies in that the clinical development has not been driven by this fact. Moreover, clinical benefit has also been observed in patients with tumors where the incidence of ras mutations has historically been very low. This clearly suggests that a second important clinical phenomenon is underway in promoting virus efficacy. As discussed extensively earlier in this review, evidence suggests that reovirus, similar to other viruses, manipulates the immune system to mount an antitumor response. As intriguing as this fact is, it only further affirms that the translation of science from "bench" to "bedside" is challenging, to say the least.

Further characterization of the activity of the virus is clearly required and it is imperative that it be in the form of a concerted effort of laboratory scientists with an interest in tumor biology, virologists, physician scientists, and academic clinicians, with a common sense of purpose. It is also critical that the patient be entirely integrated into this effort. The availability of tumor tissue to study both, the effect of this therapy and the biology behind the driving force of the cancer is absolutely essential and cannot be overstated.

It is important to note here that using viruses to target cancer is not a new phenomenon. In fact, viral approaches to cancer have been attempted for over half a century with little success (61, 62). One of the most extensively studied products is ONYX-015, an attenuated E1B-55K chimeric human group C adenovirus, which preferentially replicates within and lyses tumor cells that are p53 negative (12). Almost a decade later, another novel adenovirus mutant, ONYX-053, was created that showed that loss of E1B-55K-mediated late viral RNA export, rather than p53 degradation, restricts ONYX-015 replication in primary cells. It was experimentally proven that in contrast to the initial hypothesis, tumor cells that support ONYX-015 replication provide the RNA export function of E1B-55K (63). There was great enthusiasm for this approach when a similar product, the genetically modified adenovirus H101, made by Shanghai Sunway Biotech obtained commercial approval in China in the treatment of advanced head and neck cancer (64). Once again, despite the promises of early in vivo lab work suggesting tumor specificity, these viruses do not specifically infect cancer cells; however, they still retain some preferential cell kill for cancer cells. As of the last report, response rates were approximately doubled for H101 plus chemotherapy as compared with chemotherapy alone; however, survival information is unknown. Another limitation of this approach is that it seems to render maximum benefit when given as a direct ITu injection and the patient experiences a febrile response (65). Another virus in late-stage clinical development is Oncovex, a second generation oncolytic herpes simplex virus that expresses GM-CSF (granulocyte macrophage-colony stimulating factor) and in which deletion of infected cell protein (ICP) 34.5 provides tumor selectivity. This has been tested as a single-agent ITu therapy and in combination with radiotherapy (66, 67). It is currently in phase III clinical development to test efficacy in malignant melanoma (ongoing trial).

Other approaches using virus-mediated oncolysis have included the use of the oncolytic adenovirus ICOVIR-5 as a treatment for malignant gliomas, PV 701, an attenuated form of the Newcastle virus (68, 69) and the vesicular stomatitis virus (70–73). Even more intriguing, the measles virus is also being evaluated as an oncolytic virus, with encouraging data in breast and ovarian cancer (74–76). The reader is referred to more detailed reviews on oncolytic viruses (77–79). These examples of viral oncolytic therapy elucidate the multiple challenges that we face in developing new viral therapies for cancer, including reovirus.

Reolysin is the trade name of the therapeutic version of human reovirus formulated and developed by Oncolytics Biotech Inc.. It is a transcutaneous light blue liquid containing a purified isolate of $1 \times 10^{11} \text{TCID}_{50}$ (tissue culture infective dose) of replication competent reovirus serotype 3 Dearing strain per milliliter in a phosphate-buffered solution and is used in many of the clinical trials. The virus in its clinical formulation as Reolysin has rapidly progressed through clinical development to a phase III trial in platinum-refractory HNSCC (ongoing trial). An interesting pathway of the
clinical development of reovirus lies in metastatic CRC; where patients, whose tumors harbor a mutation in the \textit{Kras} oncogene, are ineligible to receive the anti-EGFR monoclonal antibodies, cetuximab and panitumumab (80, 81). On the basis of preclinical \textit{in vivo} data that the combination of reovirus and irinotecan is particularly synergistic in the \textit{ras}-mutant cancer cells (82), the phase I study of the combination of FOLFIRI (folinic acid, 5-fluorouracil, and irinotecan) with reovirus is currently targeting patients with a \textit{Kras} mutation, with the potential to fulfill an unmet medical need. As mentioned earlier, preclinical observations of preferential viral tropism under specific mutational status is not always replicable in human subjects. The targeting of reoviral therapy on \textit{Kras}-mutated mCRC subjects is not selected on the basis of the preclinical promises of enhanced virulence under \textit{Kras} conditions but rather due to lack of any U.S. Food and Drug Administration (FDA)-approved therapy for platinum-refractory mCRC subset of patients.

This clearly exemplifies the future of drug development: the potential of a safe and effective drug whose development is biomarker driven. The current climate of research and health care in the United States is at a crossroads with major changes expected in the manner that health care is delivered by caregivers, received by patients, and paid for by third party payers, including the governments and the private insurance plans. The success or failure of a drug being tested in clinic will be highly dependent on its absolute effectiveness in an appropriate patient profile based on a validated biomarker. This is clearly a “win win” situation for all the parties involved: the patient only receives the medication that is highly likely to benefit him/her, thereby also avoiding unnecessary toxicities, and will also bring down the cost of healthcare by paying only for effective therapies. A word of caution is appropriate here: despite the encouraging development of reovirus so far, the “proof of the pudding is in the eating”, and unless it can show improvement and patient benefit over the current standard of care in a well-conducted phase III trial, it will be relegated to the confines of history, as have many of its predecessors.

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Development of Reovirus for Cancer Therapy


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