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Risk-benefit analysis of solid tumor vs blood/metastatic cancer treatment with reovirus

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RISK-BENEFIT ANALYSIS OF SOLID TUMOR VS. BLOOD/METASTATIC CANCER TREATMENT WITH REOVIRUS

by

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ABSTRACT

In the past several years, cancer treatments using FDA approved immune checkpoint inhibitors have become more popular than common chemotherapeutic agents. However, the costs, risks, and benefits associated with these treatments are still being assessed. Currently, new therapies are being tested that utilize oncolytic viruses to treat solid tumor and metastatic cancers. Reovirus is a non-enveloped virus with a double capsid structure and a genome consisting of 10 segments of double-stranded RNA encoding eleven proteins, which has been shown to have effective oncolytic activity. There are various different strains of reoviruses that can produce cytopathic effects in mammalian host cells. Moreover, several studies have shown that reovirus can be administered in multiple ways and that administration may depend on the cancer type. This investigation examined the type 3 dearing strain of reovirus and whether different types of tumors would benefit from having a specific administration appropriate to their type such as intratumoral injections for solid tumors and intravenous administration for blood/metastatic cancers. Various

clinical trials were assessed in which reovirus was administered intratumorally, intravenously at a maximum dose of $3x10^{10}$ $TCID_{50}$, and in combinations with other cancer therapeutics. Reovirus was shown to be safe and well-tolerated across a variety of administrations and cancer morphologies. Moreover, along with its cytopathic effects, reovirus was shown to have potent immune system stimulating effects. Overall, intratumoral administration was preferred effective for solid tumor cancers while intravenous administration was preferred for blood and metastatic cancers.

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LIST OF ABBREVIATIONS

AML……...…..…Acute Myeloid Leukemia

AUC……Area under curve

BMC Cancer……BioMed Central Cancer

BZ……..Bortezomib

BU .....Boston University

CED……Convection-enhanced Delivery

CLL……..……Chronic Lymphocytic Leukemia

CR……Complete Response

DC…….……Dendritic Cell

DLT……....Dose Limiting Toxicity

EGFR……..Epidermal Growth Factor Receptor

ER…..……..Endoplasmic Reticulum/Reticular

FDA……...US Food and Drug Administration

G1…..……..Grade 1

G2……...Grade 2

IMGW…….International Myeloma Working Group

ISVP……Infectious subviral particle

JAM…….Junction Adhesion Molecule

JAM-A…..…….Junctional Adhesion Molecule-A

MTD…….Maximum Tolerated Dose

NARA...……....Neutralizing anti-retroviral antibodies

NK……...Natural Killer

OS…….Overall Survival

PD…….Progressive Disease

PFS…….Progression Free Survival

PFU…….Plaque Forming Units

PKR……..RNA-activated Protein Kinase

PR……...Partial Response

RECIST……....Response Evaluation Criteria in Solid Tumors

SD…..……..Stable Disease

T1L…..……Type 1 Lang

T3D……....Type 3 Dearing

$TCID_{50}$……Tissue culture infectious dose 50

INTRODUCTION

Cancer is the second leading cause of death in the United States after heart disease. Patients diagnosed with cancer such as local or metastatic melanoma have five-year survival rates of 98% and 17% respectively (Kohn et al. 2017). In the past several years, cancer treatments using US Food and Drug Administration (FDA) approved immune checkpoint inhibitors have become more popular as treatment options when compared to common chemotherapeutic agents (Kohn et al. 2017). However, the costs, risks, and benefits associated with these treatments are still being assessed. As seen in Table 1, although immune checkpoint inhibitors have displayed great efficacy in the treatment of cancers when other therapies have been unsuccessful, they have also been shown to not be effective at times or produce adverse events, such as the development of autoimmune disease, which increase the risk of using these types of therapies (Kohn et al. 2017).

Currently, new therapies are being tested that utilize oncolytic viruses to treat solid tumor and metastatic cancers. Oncolytic viruses specifically infect and kill cancer cells while leaving normal cells largely unharmed (Su, Jia, and Chen 2016). Oncolytic viruses can be injected into a tumor directly or inoculated into a cancer patient. Once the virus encounters and infects a cancer cell, it will replicate in that cell resulting in the release of progeny virions that can go on to infect other cancer cells once the cell is lysed (Su, Jia, and Chen 2016). Some

studies have shown that the anti-tumor efficacy of oncolytic viruses is also due to their ability to act as potent inducers of host immunity (Samson et al. 2016).

Table 1. Adverse Events Produced by Cancer Therapies. Various adverse events, both immune-derived and nonimmune-derived, occur in subjects treated with immune checkpoint inhibitors. The immune checkpoint inhibitors include pembrolizumab, ipilimumab, and nivolumab. (Kohn et al., 2017)

Adverse Event	Incidence of Grade 1 or 2 *v* Grade 3 or 4 AE[6-10]	Minimum Incidence of Grade 1 or 2 *v* Grade 3 or 4 AE	Maximum Incidence of Grade 1 or 2 *v* Grade 3 or 4 AE	Distribution[22-23]
First-line nivolumab				
Fatigue	0.329 *v* 0.013	0.269 *v* 0.003	0.399 *v* 0.033	β
Diarrhea	0.169 *v* 0.022	0.127 *v* 0.009	0.222 *v* 0.046	β
Rash	0.252 *v* 0.006	0.200 *v* 0.001	0.315 *v* 0.023	β
Nausea	0.131 *v* 0.0	0.094/0	0.178/0	β
First-line nivolumab plus ipilimumab				
Fatigue	0.310 *v* 0.042	0.251 *v* 0.022	0.378 *v* 0.071	β
Diarrhea	0.348 *v* 0.093	0.286 *v* 0.062	0.420 *v* 0.133	β
Rash	0.355 *v* 0.048	0.292 *v* 0.027	0.427 *v* 0.079	β
Nausea	0.236 *v* 0.022	0.186 *v* 0.009	0.297 *v* 0.046	β
Hypothyroid	0.147 *v* 0.003	0.108 *v* 0	0.196 *v* 0.017	β
First-line ipilimumab				
Fatigue	0.268 *v* 0.009	0.214 *v* 0.002	0.332 *v* 0.028	β
Diarrhea	0.270 *v* 0.061	0.215 *v* 0.037	0.334 *v* 0.095	β
Rash	0.309 *v* 0.019	0.250 *v* 0.007	0.377 *v* 0.042	β
Nausea	0.154 *v* 0.006	0.114 *v* 0.001	0.205 *v* 0.023	β
First-line pembrolizumab every 2 weeks				
Fatigue	0.209 *v* 0	0.158 *v* 0	0.270 *v* 0	β
Diarrhea	0.144 *v* 0.025	0.103 *v* 0.010	0.196 *v* 0.052	β
Rash	0.147 *v* 0	0.106 *v* 0	0.200 *v* 0	β
Nausea	0.101 *v* 0	0.067 *v* 0	0.146 *v* 0	β
Hypothyroid	0.097 *v* 0.004	0.064 *v* 0	0.141 *v* 0.02	β
Hepatitis	0 *v* 0.011	0 *v* 0.002	0 *v* 0.032	β
Hypophysitis	0.004 *v* 0.004	0 *v* 0	0 *v* 0.02	β
Diabetes mellitus type 1	0.0004 *v* 0.004	0 *v* 0	0 *v* 0.02	β
First-line pembrolizumab every 3 weeks				
Fatigue	0.188 *v* 0.004	0.140 *v* 0	0.246 *v* 0.02	β
Diarrhea	0.134 *v* 0.011	0.094 *v* 0.002	0.184 *v* 0.03	β
Rash	0.134 *v* 0	0.094 *v* 0	0.184 *v* 0	β
Nausea	0.112 *v* 0.004	0.076 *v* 0	0.159 *v* 0.02	β
Pneumonitis	0.014 *v* 0.004	0.004 *v* 0	0.037 *v* 0.02	β
Hepatitis	0 *v* 0.018	0 *v* 0.006	0 *v* 0.042	β
Hypophysitis	0.007 *v* 0.004	0.001 *v* 0	0.026 *v* 0.02	β
Diabetes mellitus type 1	0.004 *v* 0.004	0 *v* 0	0 *v* 0.02	β
First-line chemotherapy (dacarbazine)				
Diarrhea	0.162 *v* 0	0.116 *v* 0	0.221 *v* 0	β
Rash	0.049 *v* 0	0.025 *v* 0	0.085 *v* 0	β
Second-line nivolumab				
Fatigue	0.243 *v* 0.007	0.187 *v* 0	0.309 *v* 0.027	β
Diarrhea	0.108 *v* 0.004	0.073 *v* 0	0.155 *v* 0.021	β
Rash	0.160 *v* 0	0.116 *v* 0	0.216 *v* 0	β
Nausea	0.093 *v* 0	0.060 *v* 0	0.138 *v* 0	β
Second-line ipilimumab				
Fatigue	0.351 *v* 0.069	0.257 *v* 0.031	0.468 *v* 0.130	β
Diarrhea	0.275 *v* 0.053	0.193 *v* 0.021	0.381 *v* 0.110	β
Rash	0.191 *v* 0.008	0.124 *v* 0	0.282 *v* 0.043	β
Nausea	0.351 *v* 0.023	0.257 *v* 0	0.468 *v* 0.067	β
Second-line chemotherapy (carboplatin plus paclitaxel)				
Fatigue	0.304 *v* 0.039	0.207 *v* 0.011	0.431 *v* 0.100	β
Diarrhea	0.128 *v* 0.020	0.068 *v* 0.002	0.218 *v* 0.071	β
Nausea	0.353 *v* 0.020	0.247 *v* 0.002	0.489 *v* 0.071	β
Neutropenia	0.049 *v* 0.137	0.016 *v* 0.075	0.114 *v* 0.230	β

Abbreviation: AE, adverse event.

Reovirus is a non-enveloped virus with a double capsid structure and a genome consisting of 10 segments of double-stranded RNA encoding eleven proteins, that has been shown to have effective oncolytic activity (Kolb et al. 2015). Reovirus strains infecting humans are ubiquitous and most likely transmitted via the fecal-oral route and respiratory secretions. The prevalence of antibodies against reovirus in more than half of normal, healthy adults indicate that a majority of adults have had prior infections with reovirus, mostly likely as children (Kolb et al. 2015). Reovirus infections may be asymptomatic or associated with symptoms similar to the common cold, or diarrhea, usually in children with their first infection (Gasparinho et al. 2017). Reovirus infections in humans have not been definitively shown to cause serious illness, and were named based on the lack of association with specific disease, **r**espiratory **e**nteric **o**rphan virus. However, some studies suggest it may be associated with extrahepatobiliary atresia (EHBA) in infants (Richardson, Bishop, and Smith 1994). In studies of reovirus pathogenesis in mouse models, specific strains of reovirus cause lethal encephalitis, hydrocephalus, or biliary atresia, but only in infected neonatal mice, not when adult mice are inoculated with virus (Derrien and Fields 1999) (Wilson, Morrison, and Fields 1994).

In natural infections with reovirus, the virus most commonly enters the GI tract and undergoes limited proteolysis of the outer capsid, generating an infectious subviral particle (ISVP). Only ISVP's are capable of productively

infecting susceptible cells. ISVP's bind to receptors found on many cell types and enter cells via receptor-mediated endocytosis. Further proteolysis in endosomes results in disassembly of the outer capsid (Sakurai et al., 2017). However, unlike most other viruses, reovirus virions are not completely disassembled and viral transcription occurs within the partially uncoated virus particles in the cytoplasm. The virion contains all the enzymes required for transcribing viral mRNAs which are released through openings in the vertices of the capsid. Viral mRNAs are translated in the cytoplasm and capsid proteins assemble into icosahedral viral structures. Some of the viral mRNAs, one each of the 10 different genomic strands, are packaged into these capsids, along with newly-synthesized viral RNA polymerase. Replication of the viral genome is completed when the viral RNA polymerase uses each of the ten mRNAs as a template to transcribe the complimentary negative strand, generating the 10 double-stranded genome segments, within the capsids. Progeny virions accumulate in the cell cytoplasm until the cell lyses and they are released. Due to errors that occur during viral replication, many of the progeny virions released are defective (replication incompetent), often due to packaging errors resulting in virions that lack viral RNA, contain less than the ten genome segments or lack one or more of the ten genome segments (Martinez-Costas, Varela, and Benavente 1995) (Ogden, Ramanathan, and Patton 2011).

Reovirus has been shown to effectively kill tumor cells with an activated Ras pathway. Studies have shown this may be due to the inhibition of double-

stranded RNA-activated protein kinase (PKR) by activated Ras (Strong et al. 1998). As Figure 1 shows, when Ras is inactive, PKR is autophosphorylated in the presence of viral RNA and inhibits viral protein synthesis and replication. However, Ras activation is believed to induce downstream signals that inhibit PKR autophosphorylation and allow viral protein synthesis and replication to occur (Strong et al. 1998). One study provides evidence that MEK, a protein kinase downstream of Ras signaling pathway, is responsible for the inhibition of PKR phosphorylation (Veerapong et al. 2007). Although tumor cells with an activated Ras pathway are efficiently killed by reovirus, Sakurai and colleagues concluded that Ras-activation status does not seem to be correlated with the ability of reovirus to kill these cells (Sakurai et al. 2017). It seems that cathepsins B and L, cysteine proteases found in the endolysosomes, play a vital role in active infection and tumor-killing ability of reovirus (Sakurai et al. 2017). These cathepsins are responsible for the proteolytic disassembly of reovirus. Thus, tumors with high activities of cathepsins B and L are efficiently lysed by reovirus while tumors with low activity are usually resistant to reovirus oncolysis, likely due to inefficient endo/lysosomal escape (Sakurai et al. 2017).

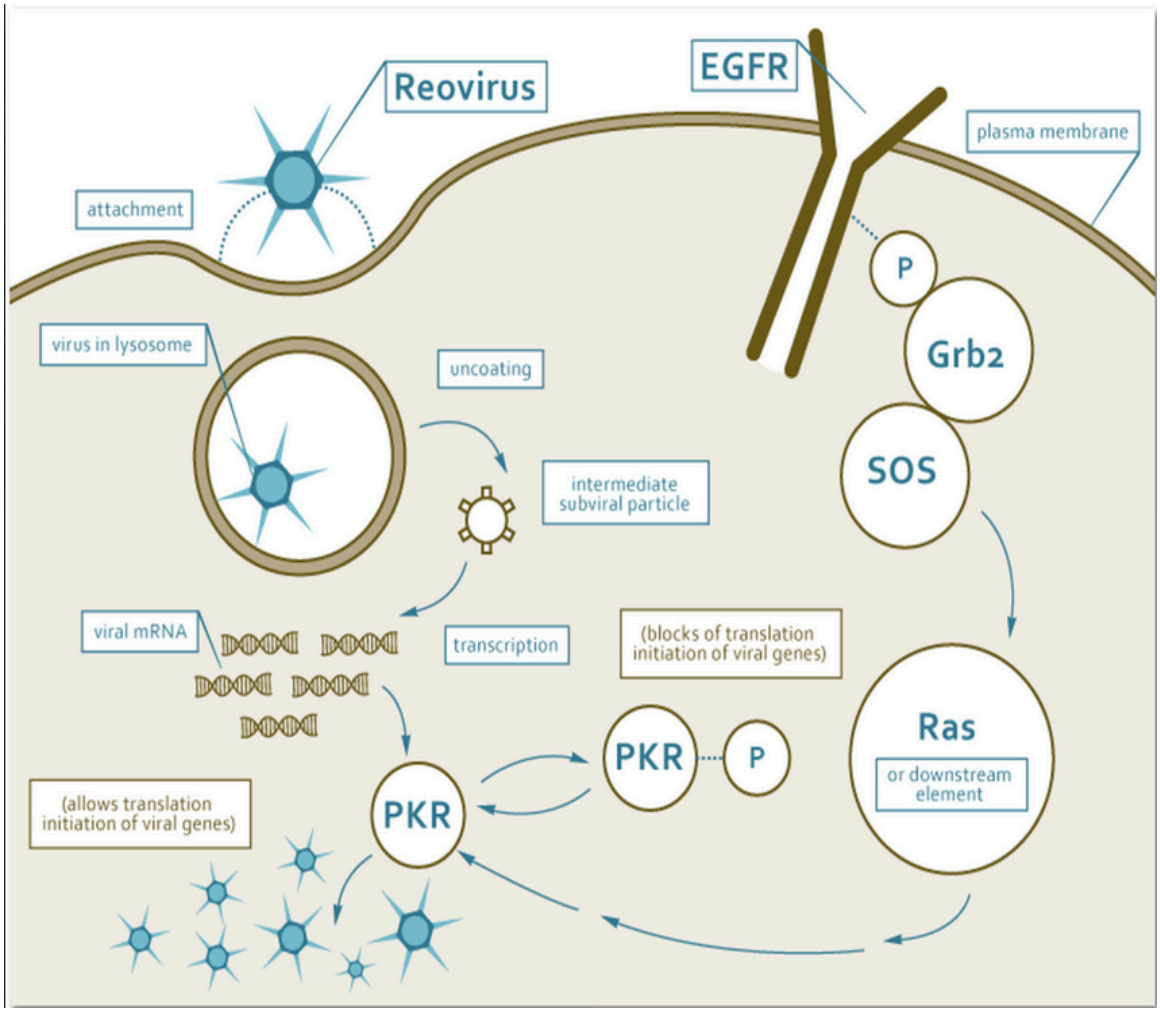

Figure 1. Ras Downstream Signaling Effects on PKR. Ras activation leads to downstream signaling that prevents PKR phosphorylation and allows for translation of viral genes. In the absence of Ras activation PKR is phosphorylated and prevents the initiation of viral gene translation. (Villalona-Calero et al. 2016)

Several studies have shown that reovirus can be administered in multiple ways and that its efficacy in killing tumor cells may be improved by varying the mode of administration depending on the cancer type. In their study of hepatocellular carcinoma, a solid tumor cancer, Samson et al. used

subcutaneous injections to deliver reovirus into the host and induce its antitumor effect (2016). Moreover, in their investigation of reovirus against lung carcinoma and melanoma, Campion and colleagues used intratumoral injections of reovirus to treat mice *in vivo* (Campion, Soden, and Forde 2016). Both studies resulted in prolongation of survival and delayed tumor growth along with upregulation of cytokines following treatment. When examining blood cancers, such as leukemia, Parrish and colleagues described how treatment of solid tumor cancers with systemic delivery of oncolytic viruses, such as reovirus, might be problematic due to decreased viral load at the tumor locale. However, for hematological malignancies, systemic delivery is ideal (Parrish et al. 2015).

Finally, although cancer therapy with reovirus has many potential benefits, there may also be some adverse outcomes. One study suggests that reovirus may lead to stimulation of helper T cells against dietary gluten leading to celiac disease (Bouziat et al. 2017). Another worry is that reovirus may not be as effective in patients with preexisting neutralizing anti-reoviral antibodies (NARA) due to previous reovirus infections (Harrington et al. 2010). In this analysis, the risks and benefits of using reovirus as a therapy for both solid tumor cancers and blood/metastatic cancers will be assessed. This investigation hypothesizes that the benefits of utilizing reovirus as an effective therapy for cancers such as melanoma, leukemia, breast, soft tissue sarcoma, etc. will outweigh the toxicities that might be incurred in patients after its administration.

SPECIFIC AIMS

1. Review the current knowledge of the mechanisms of reovirus infection and cell killing, including by apoptosis.

2. Evaluate the findings from current studies regarding the efficacy and benefits of reovirus treatments in both solid tumor cancers and blood/metastatic cancers.

3. Compare the effects of different modes of administration of reovirus on the efficacy of treatment for different types of cancer, e.g. solid tumors vs. blood cancers.

4. Investigate of the risks involved when using reovirus to treat different solid tumor and blood/metastatic cancers and how the administration influences those risks.

REOVIRUS AND REOLYSIN®

Reovirus is a member of the Orthoreovirus genus of the *Reoviridae* family, a large family that includes viruses that infect humans, animals and insects. The best-known human pathogen in this family is rotavirus, which was a widespread cause of infantile diarrhea before vaccines for it were developed. Reovirus conatains a double stranded RNA genome made up of 10 segments labeled by their size: large (L1, L2, and L3), medium (M1, M2, and M3), and small (S1, S2, S3, and S4) (Chakrabarty et al. 2014). There are various different strains of reoviruses that can produce cytopathic effects in a variety of mammalian host cells (Tyler et al. 1995). Of these strains, the type Lang (T1L) and type 3 Dearing (T3D) have been shown to induce apoptosis in cultured cells, with T3D inducing apoptosis to a greater extent than T1L (Tyler et al. 1995).

Reolysin®, also known as pelareorep, is a new investigational drug that is composed of live, replication-competent reovirus T3D strain in its purified form (Chakrabarty et al. 2014). In their study on the homogeneity and stability of the reovirus genome, Chakrabarty and colleagues found that T3D reovirus displays high genetic and genomic stability after several cycles of replication in cell cultures (Chakrabarty et al. 2014). Their investigation found 27 modifications to the genome when compared to public databases such as GenBank and concluded that these modifications gave reovirus some growth advantages. After examining the genomic stability of Reolysin®, Chakrabarty and colleagues also conducted a phylogenetic analysis using the L1 segment of Reolysin® virus

and other reoviruses, including those from different genera and host specificities (Figure 2). As expected, Reolysin® was most similar to T3D and T1L reovirus strains and also showed similarity to other mammalian reoviruses.

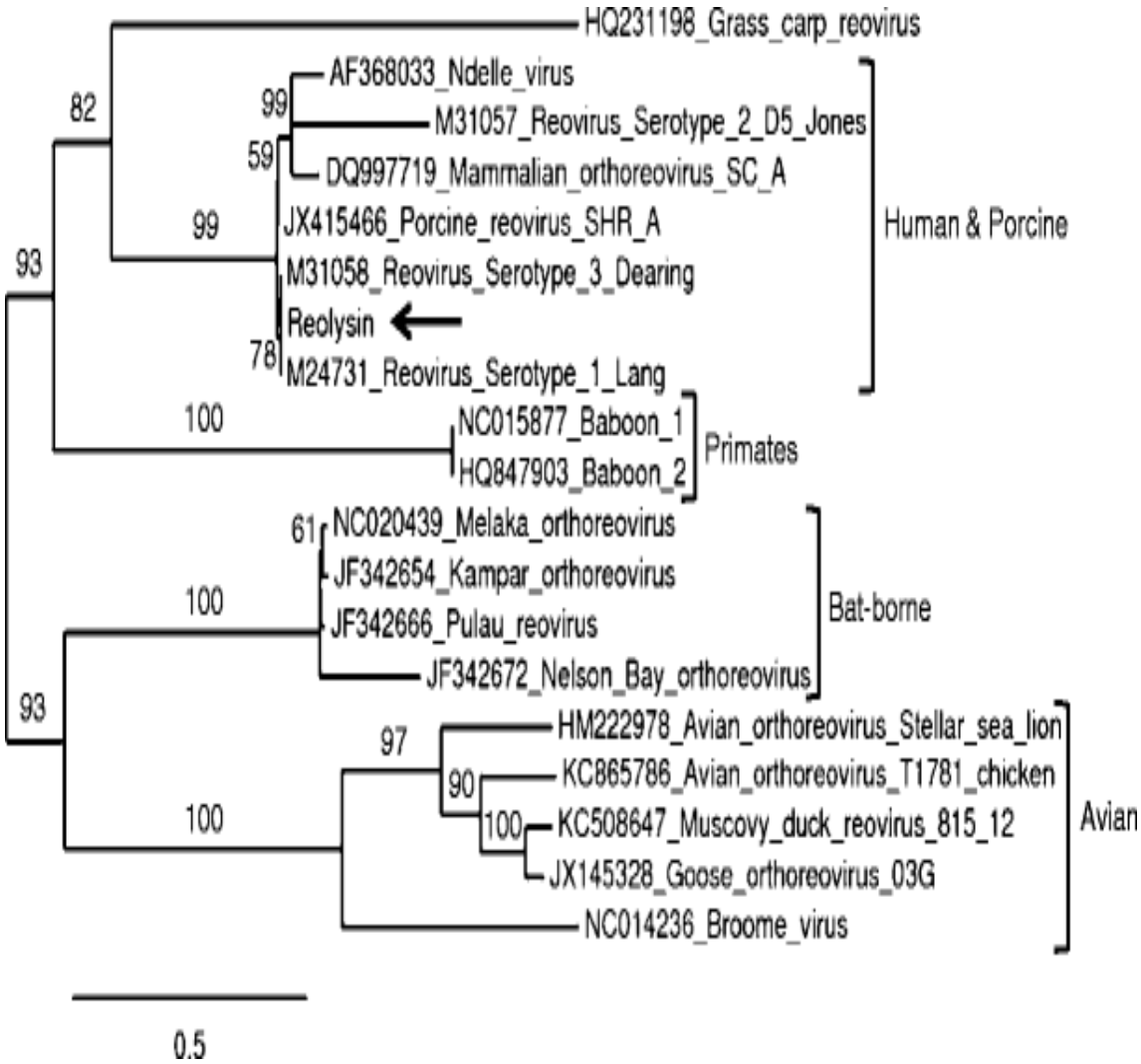

Figure 2. Phylogenetic Tree of Members from *Orthoreovirus* genus. This tree displays the evolutionary relationship between various different orthoreoviruses based on the L1 segment of their genome. There are four main clusters indicated as human and porcine, primates, bat-borne, and avian. Branch support values are shown as a percentage and the scale-bar represents 0.5 substitutions per site. (Chakrabarty et al., 2014)

MECHANISMS OF INFECTION

Previous research has demonstrated that reoviruses can preferentially infect, replicate, and induce cell death in transformed cells, particularly those with activating Ras mutations (Carew et al. 2013). However, recent studies have found that Ras activation status does not influence cell sensitivity to reovirus infection and killing and therefore should not be used as a biomarker when selecting patients for clinical trials involving reovirus (Twigger et al. 2012).

The S1 segment of the reovirus genome encodes for the viral attachment protein, $\sigma 1$, which determines reovirus tropism. $\sigma 1$ protein can bind two types of cellular receptors using independent receptor-binding domains (Barton et al. 2001). In their search for a cellular receptor for reovirus $\sigma 1$ protein, Barton and colleagues used an expression-cloning approach to identify junction adhesion molecule (JAM) as a reovirus receptor (Barton et al. 2001). T3D reovirus was shown to be dependent on JAM– $\sigma 1$ interactions to establish infection in host cells (Barton et al. 2001).

Similarly, in their investigation of the mechanisms of the anti-neoplastic effects of reovirus in multiple myeloma patients, Kelly and colleagues also demonstrated that cellular expression of junctional adhesion molecule-A (JAM-A) can determine susceptibility to reovirus infection (Kelly et al., 2015). JAM-A is an immunoglobulin superfamily protein expressed on various blood cells such as circulating neutrophils, monocytes, and lymphocytes (Kelly et al., 2015). JAM-A has many functions including the regulation of tight junctions between cells and

research on its use as a prognostic indicator of cancer progression is currently emerging (Kelly et al. 2015).

Kelly and colleagues observed how transformed cell lines with low JAM-A expression showed low levels of reovirus cell entry and replication while others with high JAM-A expression showed greater susceptibility to reovirus infection (Kelly et al. 2015). Moreover, after treatment of multiple myeloma with the proteasome inhibitor bortezomib (BZ), relapsed cells showed increased JAM-A expression leading to a greater susceptibility of these cells to reovirus infection (Kelly et al. 2015). This study highlights the potential of reovirus as a treatment when cancer cells have become resistant to a previous treatment.

MECHANISMS OF APOPTOSIS

While JAM- $\sigma 1$ interactions may help reovirus enter the host cell, investigations still need to be done to determine how reovirus exerts its effects once inside. Previous investigations have shown that cell and tissue injury induced by reovirus is associated with apoptosis (Oberhaus et al. 1997). Carew and colleagues investigated the effects of reovirus on pancreatic cancers since mutated Ras is prevalent in pancreatic cancers and reoviruses have been shown to be selective to cells with Ras activating mutations. In their investigation, Carew and colleagues demonstrated that reovirus infection led to apoptosis mediated by endoplasmic reticular (ER) stress including ER swelling, increased calcium levels in the cytosol, elevated expression of ER stress genes, and processing of

caspase-4 (Carew et al. 2013). Since previous studies had shown that processing of caspase-4 was characteristic of ER stress-mediated apoptosis, they also found that knocking out caspase-4 led to a significant reduction in reovirus-induced apoptosis (Carew et al. 2013).

Interestingly, Carew and colleagues also conducted experiments in which they treated pancreatic cancer cells with both BZ and Reolysin® *in vitro* and *in vivo* in mice and found that BZ augmented the effects of Reolysin® (Carew et al. 2013). Similarly, in their investigation of Reolysin use in multiple myeloma, Kelly and colleagues also found that reovirus infection and replication in multiple myeloma cells led to ER-stress mediated apoptosis of the infected cells (Kelly et al. 2012). Cotreatment with Reolysin® and BZ in multiple myeloma also showed elevated levels of ER stress due to the increased accumulation of viral and ubiquitinated proteins (Kelly et al., 2012).

LOCAL/INTRATUMORAL REOVIRUS TREATMENT – SOLID TUMORS

In their phase I trial treating patients with advanced solid tumors, Morris and colleagues used a dose-escalation design with percutaneous, intralesional administration of Reolysin® (Morris et al., 2013). Nineteen patients with different solid tumor cancers, including soft tissue sarcoma, head and neck cancer, and breast cancer were enrolled in the study as seen in Table 2 (Morris et al., 2013). Intralesional administration was conducted in an outpatient setting under sterile

conditions and patients were monitored for two hours following the procedure (Morris et al. 2013).

Table 2. Patient Demographics and Tumor Diagnoses in a Study of Intralesional Reovirus Administration. The characteristics of the patients enrolled in this study are shown. This table also displays the previous therapies that the patients may have had prior to enrollment. (Morris et al, 2013)

Patient characteristics	**No. patients**
Total No.	19
Age (years)	
Median	51
Range	27–75
Male/Female	9/10
Primary tumor site	
Soft tissue sarcoma	5
Head and neck	4
Melanoma	4
Breast	3
Other	3
Eastern Cooperative Oncology Group Performance Status	
0	12
1	7
Prior surgery	16
Prior radiotherapy	10
Prior chemotherapy	15
Median Regimens (range)	2 (0–5)

In order to evaluate tumor responses to Reolysin® treatment, patients were monitored for 14 weeks, with responses being evaluated once a week for the first 6 weeks and then at weeks 10 and 14 afterwards. Tumors were manually measured using calipers and clinical responses were determined by Response Evaluation Criteria in Solid Tumors (RECIST) for progressive disease (PD), stable disease (SD), partial response (PR) and complete response (CR) (Therasse et al. 2000). The best target tumor response at/after 6 weeks follow-up was CR in one (5.3%), PR in two (10.5%), SD in four (21.1%) and PD in ten (52.6%) patients, indicating a statistically significant treatment efficacy for the local tumors (Morris et al. 2013). However, no significant anti-tumor activity was found for tumor lesions in sites remote from the viral administration site. This is believed to be due to the NARA response that prevents the spread of virus.

One major finding in the Morris study was that percutaneous intralesional administration of Reolysin® to oncology patients with a variety of cancers and was well-tolerated and safe. The chief toxicities observed were mild, including nausea, vomiting, diarrhea, injection site erythema, and fever/chills (Morris et al. 2013). Moreover no dose limiting toxicity (DLT) was found, even at the highest dose used of $1x10^{10}$ plaque forming units (PFU), and thus the maximum tolerated dose (MTD) could not be defined (Morris et al. 2013).

In their phase I clinical trial of genetically unmodified, T3D reovirus and its use in malignant gliomas, Forsyth and colleagues also used intratumoral injections to help define a DLT/MTD and examine tumor responses to reovirus.

Twelve patients with a median age of 53.5 years and history of recurrent malignant glioma were enrolled in the study. As shown in Table 3, the patients had varying brain tumor histologies and various prior therapies including radiotherapy and chemotherapy (Forsyth et al. 2008).

Table 3. Demographics of Patients with Malignant Glioma in an Intratumoral Injection Trial . The characteristics of the patients enrolled in this study are shown. This table also displays the previous therapies that the patients may have had prior to enrollment and the distribution of patients among the doses. (Forsyth et al., 2008)

	# Patients	%
Gender		
Male	7	58
Female	5	42
Performance status (KPS)		
60	3	25
70	2	17
80	2	17
90	5	42
Histology at first diagnosis of brain tumor		
Oligoastrocytoma grade II	1	8
Anaplastic oligoastrocytoma	1	8
Anaplastic astrocytoma	3	25
Glioblastoma multiforme	7	58
Histology of recurrence prior to Reovirus administration		
Anaplastic astrocytoma	2	17
Anaplastic oligoastrocytoma	1	8
Glioblastoma multiforme	9	75
Prior therapy		
Radiotherapy (60 Gy)	2	100
Adjuvant chemotherapy	10	83

Other medications at baseline		
Corticosteroids	6	50
Anticonvulsants	12	100
Dose ($TCID_{50}$)		
10^7	3	25
10^8	6	50
10^9	3	25
Surgery at Recurrence post administration		
Resection	6	50
None	6	50

Tumor responses were evaluated using standard criteria with one patient having SD, ten patients having PD, and one patient not evaluable for response due to a small hemorrhage leading to surgery (Forsyth et al., 2008). Moreover several patients had longer-than-expected survivals and one remains a long-term survivor, but that may have been due to selection criteria for the study rather than reovirus treatment (Forsyth et al., 2008).

Similar to the previous study by Morris, the major finding in this research study is that administration of reovirus intracerebrally, directly into brain tumors of patients with malignant glioma, was safe and well-tolerated at the doses used (Forsyth et al., 2008). The maximum dose used was $1x10^9$ tissue culture infectious dose 50 ($TCID_{50}$) and the most common adverse event seen was a deterioration of motor strength that was due to tumor progression rather than treatment with reovirus (Forsyth et al., 2008).

In another phase I clinical trial examining reovirus and its use in malignant gliomas, Kicielinski and colleagues used a different technique to administer Reolysin® to patients. This investigation used an appropriate placement of catheters to allow for direct infusion of reovirus into the tumor (Kicielinski et al., 2014). This delivery method is called convection-enhanced delivery (CED) and relies on sustained, low-pressure infusion with a catheter to allow for enhanced delivery to the site of interest while limiting systemic side effects and enhancing efficacy (Kicielinski et al., 2014).

Eighteen patients were enrolled in this trial, but only fifteen were treated. Three patients displayed pathologies with grade 3 tumors (anaplastic astrocytomas) and the remaining patients displayed grade 4 tumors (glioblastoma multiforme) (Kicielinski et al. 2014). This investigation used a does-escalation method with doses ranging from $1x10^8$ to $1x10^{10}$ $TCID_{50}$.

Tumor responses to the treatment were analyzed using standard criteria and at the best response during the study period 10 patients had SD, 1 had PR, and 4 had PD. By the time of the study's discontinuation, 2 patients had SD, 1 had PR, and 12 had PD (Kicielinski et al. 2014). Although many of the SD patients eventually returned to PD, the study demonstrates that treatment with reovirus managed to help a few patients, showing its potential to combat malignant gliomas. Moreover, several of the patients that participated in the study experienced prolonged survival, which was likely due to the treatment with reovirus.

The main goal of this experiment was to analyze the safety and tolerability of Reolysin® treatment. Even with a maximum dose of $1x10^{10}$ $TCID_{50}$, no DLT was reached and so no MTD was discovered. The most common adverse events experienced were convulsions and seizures which occurred in 2 patients at the highest dose level, but were determined to be unrelated to reovirus and the infusion delivery method (Kicielinski et al., 2014). CED of reovirus was well tolerated by the patients, but further investigations into reovirus at the highest dose level should be done to confirm the safety of this protocol.

In another phase I clinical trial, Harrington and colleagues combined radiotherapy with intratumoral reovirus treatment in patients with advanced solid tumor cancers. Twenty-three patients with advanced or metastatic solid tumors were treated in this study. These patients had varying tumor types including melanoma, head and neck cancer, lung cancer, pancreatic cancer, and colorectal cancer. The median age for patients was 58.6 with ages ranging from 38-75 (Harrington et al., 2010).

The study was conducted in two stages. In the first stage, radiotherapy was given as local tumor irradiation at a dose of 20 Gy in five consecutive daily fractions along with two intratumoral injections of Reolysin® at doses between $1x10^{8}$ to $1x10^{10}$ $TCID_{50}$ (Harrington et al., 2010). In the second stage, patients received local tumor irradiation at a dose of 36 Gy in 12 fractions over 16 days with two, four, or six doses of T3D reovirus at doses of $1x10^{10}$ $TCID_{50}$ (Harrington et al., 2010). A previous study showed *in vitro* and *in vivo* data that provided

evidence for a synergistic effect between reovirus and radiotherapy that prompted this investigation (Twigger et al., 2008).

Overall, treatment was well-tolerated in all cohorts of the study. The most common toxicities observed included pyrexia, flu-like symptoms, vomiting, asymptomatic lymphopenia, and neutropenia (Harrington et al., 2010). However, researchers determined that neither reovirus treatement nor radiotherapy were responsible for these toxicities. No DLT was reached in this study and thus no MTD was found. Like the previous studies, this investigation demonstrated that intratumoral injections of reovirus at a dose of $1x10^{10}$ $TCID_{50}$ were well-tolerated and recommended for future studies.

Of the twenty-three patients treated, fourteen were evaluable for treatment response. From the patients in the first stage, 2 out of 7 had PR and the other 5 had SD. From patients in the second stage, 5 out of 7 had PR and the other 2 had SD (Harrington et al. 2010). However, it is not possible to draw conclusions on the influence that Reolysin® had on the tumor responses. Overall, this investigation demonstrated the possibility of combining reovirus with current cancer therapies such as radiation and further supports its safety.

INTRAVENOUS REOVIRUS TREATMENT – SOLID TUMORS

While intratumoral administration of T3D reovirus for solid tumor cancers has been shown several times to be safe and well-tolerated, researchers also began investigating the possibility of administering reovirus intravenously. In their

phase I clinical trial, Kolb and colleagues examined the effects of treating children with extra-cranial solid tumors with Reolysin®. For 5 consecutive days every 28 days reovirus was administered intravenously to patients in one of three dose levels including $3x10^8$ $TCID_{50}$/kg of weight, $5x10^8$ $TCID_{50}$/kg of weight, and $5x10^8$ $TCID_{50}$/kg of weight + 50 mg/m^2/day of oral cyclophosphamide for 21 consecutive days every 28 days (Kolb et al., 2015).

Twenty-nine patients were enrolled in the study but only twenty-four were evaluable. Patients had a median age of 12.5 years with ages ranging from 3 to 20.2 years and were of different races as seen in Table 4. Moreover, patients displayed varying tumor types, including Wilms tumor, neuroblastoma, osteosarcoma, retinoblastoma, etc. (Kolb et al., 2015). Tumor response assessments were done with RECIST criteria.

Table 4. Patient Demographics in a Study of Intravenous Administration of Reovirus. The characteristics of the patients enrolled in this study are shown. This table also displays the previous therapies that the patients may have had prior to enrollment. (Kolb et al., 2015)

Characteristic	**Number (%)**
Age (years)	
Median	12.5 years
Range	3.0–20.2 years
Sex	
Male	19 (67.9)
Female	9 (32.1)
Race	
White	18 (64.3)
Asian	2 (7.1)
Native American	1 (3.6)

Characteristic	**Number (%)**
Pacific Islander	1(3.6)
Black or African American	4 (14.3)
Ethnicity	2 (7.1)
Non-Hispanic	24 (85.7)
Hispanic	4 (14.3)
Diagnosis	
Alveolar rhabdomyosarcoma	2 (7.1)
Chondroblastic osteosarcoma	1 (3.6)
Clear cell sarcoma	1 (3.6)
Desmoplastic small round cell tumor	1 (3.6)
Embryonal rhabdomyosarcoma	3 (10.7)
Ewing sarcoma	3 (10.7)
Germ cell tumor	1 (3.6)
Hemangiosarcoma/angiosarcoma	1 (3.6)
Hepatoblastoma	2 (7.1)
Neoplasm, malignant/Tumor, malignant, NOS	1 (3.6)
Wilms tumor	3 (10.7)
Neuroblastoma	2 (7.1)
Osteosarcoma	3 (10.7)
Retinoblastoma	1 (3.6)
Synovial sarcoma	2 (7.1)
Prior Therapy	
Chemotherapy Regimens	
Median	3
Range	1–8
Number of patients with prior radiation therapy	20 (71.4)

Overall, the study concluded that reovirus can be administered safely to children and an MTD was not reached. Interestingly, a DLT was reported for one patient at a dose level of $3x10^8$ $TCID_{50}$/kg and the patient went into respiratory

failure and died. However, this adverse event was attributed to progressive disease and unlikely caused by the treatment. Another DLT was reported for one patient with synovial sarcoma at a dose level of $5x10^8$ $TCID_{50}$/kg. The patient experienced increasing shortness of breath and a deep venous thrombosis, which were attributed to Reolysin® treatment and progressive disease. Other common toxicities included abdominal pain, chills, diarrhea, fatigue, fever, and headaches (Kolb et al., 2015).

When looking at tumor responses, the study did not report any CR or PR. Three patients were reported to have SD at one point in the study. Cyclophosphamide is an immunosuppressive drug that the researchers hoped would increase intratumoral virus levels and tumor response. While tumor response may not have been affected much by the combined treatment (or reovirus alone), no DLTs for the combined therapy were reported and viral clearance was not impacted (Kolb et al., 2015). This suggests that this combined therapy was safe and could be used in future studies of Reolysin® in patients.

INTRAVENOUS REOVIRUS TREATMENT – METASTATIC CANCERS

In their investigation of intravenous administration of Reolysin® in patients with advanced solid tumors, Gollamudi and colleagues used six dose cohorts to evaluate the safety of systemic treatment of Reolysin®. Reolysin® was given as a one-hour intravenous infusion every 28 days with the lowest dose cohort being

$1x10^8$ $TCID_{50}$ and the highest dose cohort being $3x10^{10}$ $TCID_{50}$ (Gollamudi et al., 2010).

Eighteen patients were enrolled in the study with a median age of 57 years and ages ranging from 40-72 years. Patients had various cancers, including colon cancer, ovarian cancer, prostate cancer, and breast cancer. Since they were being treated with live virus, patients were asked to stay at home as much as possible in the first five days of treatment and wear masks for 2 weeks when around others (Gollamudi et al., 2010).

The common toxicities seen in all patients included fever, chills, headache, cold-like symptoms, and fatigue, among many others, and are displayed in Table 5. None of the toxicities required any dose reduction or management as they went away with time (Gollamudi et al., 2010). Overall, no DLT was reached indicating that intravenous infusion of reovirus was safe and fairly well-tolerated, even at the maximum dose of $3x10^{10}$ $TCID_{50}$. This investigation highlights an important point on the safety of using live, replication-competent reovirus and its potential for future therapies.

Of the 18 patients enrolled in the study, 16 were evaluable for tumor responses using RECIST criteria. One patient with breast cancer experienced PR with 34% reduction in tumor burden and 12 other patients experienced SD as their best response. Interestingly, no relationship was found between a higher dose level and higher incidence of clinical benefit. Overall, this investigation demonstrated the effectiveness and safety of cancer therapy with Reolysin®,

even though all the patients experienced some toxicity believed to be related to regular infection with reovirus (Gollamudi et al. 2010).

Table 5. Adverse Effects Experienced by Patients Across Doses Following Intravenous Reovirus Treatment. The various adverse events experienced by patients in this study are shown. Abbreviations: Grade 1 (G1) and Grade 2 (G2). (Gollamudi et al. 2010)

Dose Level	**1**		**2**		**3**		**4**		**5**		**6**	
$TCID_{50}$	**1×10^8**		**3×10^8**		**1×10^9**		**3×10^9**		**1×10^{10}**		**3×10^{10}**	
	G1	**G2**	**G1**	**G2**	**G1**	**G2**	**G1**	**G2**	**G1**	**G2**	**G1**	**G2**
Fever	2									1	3	
Chills	1		1		1				1			
Myalgia			1				2		3		2	
Headache	2				1				1		1	
Sore throat/Nasal fullness	2						1		2			
Fatigue			2	1			1		2		1	
Dehydration	1											
Nausea	1		1						1		2	
Vomiting			1				1		1			
Diarrhea	1		3				1					
Constipation			1						1			
Bloating									1			
Anorexia			1				1		1		1	
Dysgeusia											1	
Skin rash									1			

In another phase I study of intravenous administration of reovirus in patients with advanced cancers, Vidal and colleagues used Reolysin® to examine the safety and efficacy of intravenous administration. Patients were divided into

eight cohorts, with the first cohort given a single dose every four weeks at $1x10^8$ $TCID_{50}$ and the last cohort given doses for five consecutive days every four weeks at $3x10^8$ $TCID_{50}$ (Vidal et al., 2008).

Thirty-three patients with various malignant diagnoses were enrolled in the study and had a median age of 59.5 with ages ranging from 32-80. Cancers included head and neck, prostate, colorectal, pancreatic, and other types.

Similar to the previous study by Gollamudi et al., treatment was well tolerated and no DLT was reached. A dose of $3x10^{10}$ $TCID_{50}$ for five consecutive days every four weeks was defined as the MTD as it was the highest dose available for administration (Vidal et al., 2008). The most common adverse effects seen were fever, chills, fatigue, nausea, and vomiting. Overall the protocol was shown to be safe and recommended for future investigations. Moreover, although no objective responses using RECIST criteria were made, there were indications of antitumor activity including in 8 patients with radiologic evidence of stable disease.

In their phase II trial examining the antitumor effect of Reolysin® in patients with metastatic melanoma, Galanis and colleagues also used intravenous administration. Reolysin® was given at a dose of $3x10^{10}$ $TCID_{50}$ for five consecutive days every four weeks (Galanis et al., 2012)

Twenty-three patients were enrolled in the study and twenty-one were eligible for treatment. Patients had a median age of 65 years and with ages

ranging from 22-80 years. Many of them had received prior therapies before enrolling in the study.

Overall, the treatment was well-tolerated with a few severe (grade 3-4) treatment-related toxicities including fatigue, lymphopenia, and hyponatremia. The most common toxicities were mild (grade 2 or less) and included nausea, fever, and anemia (Galanis et al., 2012).

Tumor responses were evaluated using RECIST criteria and no PR or CR were observed. However one patient did show extensive tumor necrosis in two of their metastatic lesions that had to be surgically removed. In this investigation 15 patients had biopsies done to evaluate for reovirus infection. Of the 15, 13 contained metastatic tumors, and in 2 out of the 13, productive reovirus replication was detected (Figure 3). Interestingly the median progression-free survival rate of patients enrolled in this study was 45 days, but the two patients with reovirus replication in their metastatic tumors survived for 80 and 87 days (Galanis et al., 2012).

Although this study does not support the use of intravenous administration of reovirus as a monotherapy for malignant melanoma, it does highlight its ability to infect and replicate in melanoma metastases. One factor that may have resulted in this low treatment efficacy is the presence of NARA in patients. Researchers saw a multiple fold increase in neutralizing antibodies against reovirus in all patients treated in the study so a decrease in efficacy due to NARA may have been possible. In general, intravenous administration of reovirus may

prove to be advantageous in combatting metastatic cancer in future investigations.

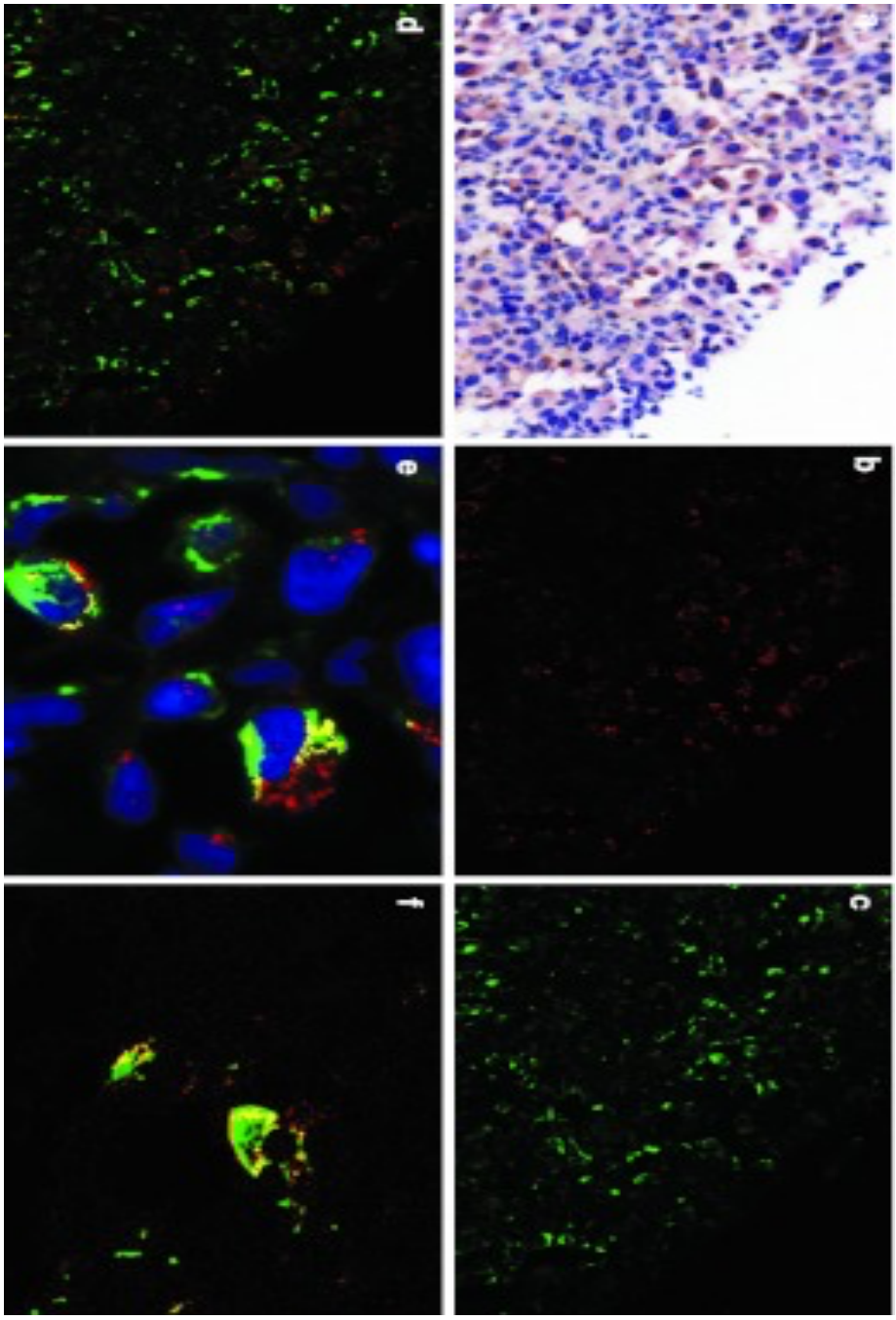

Figure 3. Expression of Reoviral Proteins in Post-treatment Biopsies. (A) Hemotoxylin and eosin stain. (B) Reovirus stain (red). (C) Tubulin stain (green).

(D) Cells that show coexpression of reoviral proteins and tubuin are fluorescent yellow. (E) Higher magnification of an area of double positive cells indicating active reovirus infection. (F) Coexpression of reovirus (red) with p38 (green) are fluorescent yellow. (Galanis et al. 2012)

REOVIRUS IN COMBINED THERAPIES – METASTATIC CANCERS

As the previous studies have shown, Reolysin® alone can infect tumors and produce some beneficial effects in cancer patients with both primary and metastatic tumors. While on its own it may not possess the desired strength needed to promote its use as a cancer monotherapy, researchers recognized its potential and began investigating its use in combined therapies.

In a phase I study combining intravenous administration of reovirus with docetaxel, Comins and colleagues investigated the safety and efficacy of using this combination therapy in patients with advanced cancer. Docetaxel is an antineoplastic drug that disrupts the normal process of microtubule assembly and disassembly. Reovirus had previously been shown to associate with microtubules and thus docetaxel may play a role in making viral replication more efficient (Comins et al. 2010). The investigation used a dose-escalation design with three cohorts. Each cohort was given 75mg/m^2 docetaxel through intravenous infusion for one hour every three weeks and intravenous infusion of T3D replication-competent reovirus (Reolysin®) for five consecutive days every three weeks at doses of either $3x10^9$ $TCID_{50}$, $1x10^{10}$ $TCID_{50}$, or $3x10^{10}$ $TCID_{50}$.

Twenty-five patients were enrolled in the study with a median age of 60 years and ages ranging from 32-77 years and twenty-three patients completed at

least 1 cycle of treatment. The study included patients with advanced or metastatic solid tumors, including melanoma, prostate, pancreatic, stomach, and breast, for which no curative standard therapy was available (Comins et al. 2010).

Overall the treatment was well-tolerated with common side effects including diarrhea, fatigue, fever, chills, and neutropenia. Moreover there were six grade 4 adverse effects, with four of them occurring in the highest dose cohort. However, these four were attributed to docetaxel therapy alone (Comins et al., 2010). One DLT was reached due to grade 4 neutropenia and while MTD was not reached the study authors recommended a dose of $3x10^{10}$ $TCID_{50}$ with 75 mg/m^2 of docetaxel for future studies (Comins et al., 2010).

Sixteen patients were eligible for response assessment that was carried out according to RECIST criteria. One CR was reported in the liver of a patient with metastatic breast carcinoma. Three patients had evidence of PR and ten other patients had evidence of SD. However it is impossible to determine whether responses were due to either agent alone or through combination. Three patients had post-treatment biopsies taken and reovirus proteins were detected in all of these biopsies, indicating that reovirus was transported to sites of metastases (Comins et al., 2010). In general, this investigation highlights the potential benefit of administering combined treatments of reovirus with current antineoplastic drugs.

In another trial examining the combined therapy of Reolysin® with carboplatin and paclitaxel chemotherapy, Karapanagiotu and colleagues used a dose-escalation design to determine the safety and DLT/MTD of the combined treatment in patients with advanced or metastatic malignancies. Patients were given 175 mg/m^2 of paclitaxel and area under curve (AUC) 5 of carboplatin through intravenous infusion for one day every three weeks. Three dose cohorts of either 3x10^9, 1x10^{10}, or 3x10^{10} $TCID_{50}$ of reovirus were used to administer to patients intravenously for one hour for five consecutive days every three weeks (Karapanagiotou et al., 2012)

Thirty-four patients were enrolled in the study, while thirty-one received treatment. Patients had a median age of 60 years with ages ranging from 27-79 years and varying cancer diagnoses such as squamous cell cancer of head and neck, melanoma, and sarcoma.

Treatment was well-tolerated by patients with the most common toxicities including blood cytopenias, nausea, vomiting, fatigue, diarrhea, fever, chills, and muscle pain (Karapanagiotou et al., 2012). Some grade 3/4 toxicities were also observed, including neutropenia, asymptomatic lymphopenia, and anemia. However, no relationship between reovirus dose level and incidence or grade of symptoms was found. No MTD was reached for reovirus treatment and many of the adverse events seen were not uncommon in chemotherapy alone.

To assess tumor responses to treatment RECIST criteria was used. Of the 31 patients treated, 26 had received at least two cycles of treatment and were

evaluable for tumor response in their primary tumors. One patient had CR, 6 patients had PR, 9 patients had SD, and 8 patients had PD. The other 2 patients were not evaluable by RECIST, but had major clinical responses in radiation pretreated lesions and one of them even had a nearly complete disappearance of disfiguring lesions (Karapanagiotou et al., 2012). Overall, this combined treatment was shown to be safe and effective in treating patients with advanced malignancies. However, this study was limited in determining efficacy of treatment for metastatic cancers by not examining tumor response in metastases.

Lolkema and colleagues investigated the safety and efficacy of combining intravenous reovirus treatment with gemcitabine to attenuate the neutralizing antibody response against reovirus. Previous studies in mice with human colon cancer xenografts showed the synergy produced by *in vivo* treatment with Reolysin® and gemcitabine with pathologic complete remission in 4/5 mice (Lane et al. 2007). Gemcitabine was administered through 30-minute intravenous infusion at a dose of 1000 mg/m^2 on days 1 and 8 of a three-week cycle. Initially, Reolysin® was planned to be administered for five consecutive days every three weeks, but the first two patients treated experienced DLT and protocol was changed to administer Reolysin® at day 1 only of each three-week cycle. Four escalating dose levels were used with the lowest being $1x10^9$ $TCID_{50}$ and the highest being $3x10^{10}$ $TCID_{50}$ (Lolkema et al., 2011).

Sixteen patients were enrolled in the study with ages ranging from 36-72 years. Patients were diagnosed with varying solid malignancies, including colorectal, breast, squamous cell carcinoma, and fibrosarcoma.

The most common adverse effects reported include pyrexia, nausea, diarrhea, vomiting, chills, and increased ALT. These were generally mild or moderate. As mentioned previously, two patients experienced DLT at a dose of $3x10^{10}$ $TCID_{50}$ of reovirus for 5 consecutive days every three weeks with 1000 mg/m^2 of gemcitabine on days 1 and 8. These DLTs were due to a rise in liver enzymes, ALT and AST, which is known to be a common side effect of gemcitabine (Lolkema et al. 2011). Although an MTD was not determined, study authors recommend a dose of $1x10^{10}$ $TCID_{50}$ of reovirus on day 1 and 1000 mg/m^2 of gemcitabine on days 1 and 8 of a three-week cycle for future trials.

Ten patients were evaluable for tumor response and had completed at least 2 cycles of treatment. One patient with metastatic nasopharyngeal carcinoma had PR according to RECIST and another with breast cancer showed decreased tumor size. Six other patients showed SD. Gemcitabine was used to attenuate the NARA response, which researchers hoped would allow reovirus to spread more easily to other tissues. Overall, the study showed a modest efficacy for the combined treatment, but warrants further study for ideal dose levels and to elucidate mechanisms for the efficacy.

REOVIRUS IN COMBINED THERAPIES – RAS METASTATIC CANCERS

The previous studies examined patients with various tumor types, not excluding patients with cancers unrelated to activating mutations in Ras. As mentioned before some studies concluded that Ras activation status did not affect infectivity of reovirus, while others state that Ras activation plays a role in reovirus infectivity and replication. One investigation discovered that the majority of cancers of the exocrine pancreas were related to activating mutations in K-ras genes (Almoguera et al., 1988). In their phase 2 trial of Reolysin® combined with carboplatin and paclitaxel, Noonan and colleagues investigated the efficacy of this combined therapy in patients with metastatic pancreatic adenocarcinomas (2016). All patients received intravenous infusion of 175 mg/m^2 of paclitaxel over three hours followed by AUC5 carboplatin over thirty minutes on day 1 of a three-week cycle. Patients in Arm A also received intravenous infusion of Reolysin® at $3x10^{10}$ $TCID_{50}$ for five consecutive days every three weeks while patients in Arm B did not (Noonan et al., 2016).

Seventy-three patients with a median age of 64 years and ages ranging from 39-84 were treated in the study. Thirty-six patients were randomized to Arm A and the other thirty-seven to Arm B. Patients had varying metastases to the liver, lung, peritoneum, and other sites with about half the patients having metastases in three or more sites (Noonan et al., 2016).

The treatment was well-tolerated by the patients with no significant differences in adverse effects between each arm. However there were two

occurrences of lupus nephritis attributed to treatment with Reolysin® and both were resolved after discontinuing virus treatment.

Out of the 73 patients treated, only 5 had progression free survival (PFS) and the remaining 68 had disease progression and/or death. The study found no significant difference in PFS between treatment arms with Arm A having a median PFS of 4.9 versus 5.2 months for Arm B. Furthermore, no significant difference in overall survival (OS) was found between treatment arms with Arm A having a median OS of 7.3 months versus 8.8 for Arm B. Looking at tumor response, seven patients in each arm displayed PR. Moreover SD was seen in 53% of patients in Arm A and 49% of patients in Arm B. Overall, combination therapy of Reolysin® with carboplatin and paclitaxel did not improve PFS in patients with pancreatic adenocarcinoma when compared to treatment with carboplatin and paclitaxel alone (Noonan et al., 2016). Interestingly, this study provides some evidence that K-ras status does not influence tumor response or survival outcomes in patients treated with reovirus combined with chemotherapeutics.

In another investigation evaluating Reolysin® combined with paclitaxel and carboplatin, Villalona-Calero and colleagues treated KRAS-activated tumors in patients with metastatic or recurrent non-small cell lung cancer (2016). Patients were given intravenous administration of AUC 6 mg/ml/min of carboplatin and 200 mg/m^2 of paclitaxel on day one of the three-week cycle. Reolysin® was also administered intravenously at a dose of 3x10^{10} $TCID_{50}$ for five consecutive days

every three weeks (Villalona-Calero et al. 2016). Compared to previous studies with this combined therapy, this investigation started by using slightly higher doses for carboplatin and paclitaxel. After the first two patients treated with these starting doses experienced unacceptable toxicities, doses for carboplatin and paclitaxel were reduced to AUC5 and 175 mg/m^2 respectively for all patients for the remainder of the study, matching the doses of previous studies (Villalona-Calero et al., 2016).

Thirty-seven patients with median age of 65 years and ages ranging from 47-82 years were treated in the study. Patients had varying molecular alterations including 20 with KRAS mutations, 10 with epidermal growth factor receptor (EGFR) amplifications, 3 with EGFR mutations, and 4 with BRAF-V600E mutations.

The most frequent moderate to severe adverse effects observed included fatigue, neutropenia, diarrhea, nausea, and vomiting. Overall, the treatment was well tolerated and had a good safety profile. Chemotherapy was further reduced for 6 patients due to adverse events and Reolysin® dose was reduced for only 1 patient.

Out of the thirty-seven patients treated, only 35 were evaluable for a response. According to RECIST criteria 11 out of 35 showed PR. When comparing tumor responses according to molecular alteration, 5 out of 18 with KRAS mutation showed PR, 3 out of 10 with EGFR amplification mutation showed PR, and 2 out of 4 with BRAF mutation showed PR. These results

indicated that the type of KRAS-activating mutation in the tumor did not affect tumor response. Moreover because the study lacked a control group with non-KRAS-activated tumors the study could not confirm or reject the use of Ras as a predictor for Reolysin® activity (Villalona-Calero et al., 2016).

INTRAVENOUS REOVIRUS TREATMENT – BLOOD CANCERS

While the safety and efficacy of reovirus treatment in solid tumor cancers has been shown, its potential use as a therapy for blood cancers such as myeloma and leukemia is still being investigated. Previous studies have shown that reovirus can induce ER-stress mediated apoptosis in myeloma cells, thus demonstrating a benefit in attempting to treat some blood cancers with intravenous reovirus.

In their phase I clinical trial, Sborov and colleagues investigated the safety and efficacy of utilizing intravenous administration of Reolysin® in patients with relapsed multiple myeloma (2014). Two dose cohorts at either $3x10^9$ $TCID_{50}$ or $3x10^{10}$ $TCID_{50}$ were used in which all patients were given intravenous infusion of Reolysin® for five consecutive days in four-week cycles.

A total of twelve patients with a median age of 61 years and ages ranging from 48-77 years were enrolled in the study. All patients had received prior treatment with lenalidomide and bortezomib (Sborov et al., 2014).

Overall the treatment was well-tolerated and no DLT was reached. Three patients experienced grade 3 neutropenia and two experienced grade 3

leukopenia. Grade 2 toxicities included leukopenia, anemia, neutropenia, thrombocytopenia, and myalgias (Sborov et al., 2014).

Response assessment was conducted according to International Myeloma Working Group (IMWG) Uniform response criterion (Durie et al., 2006). The best response observed was stable disease. In cycle 1, 5 patients displayed decreased myeloma proteins, 3 had slight increases, and the other 4 showed signs of progressive disease. Seven patients had adequate tissue samples for pre- and post-treatment bone marrow biopsies and in all seven reovirus RNA was found. Moreover reovirus protein was also found in five of these biopsies indicating evidence of reovirus cell entry and replication (Sborov et al., 2014). In general, this investigation demonstrated that treatment of multiple myeloma with a $3x10^{10}$ $TCID_{50}$ dose of Reolysin® was safe, with some moderate adverse effects, but only showed a modest efficacy. While reovirus was shown to infect cells, replication and apoptosis were limited.

IMMUNE SYSTEM EFFECTS OF REOVIRUS

While infecting cancer cells and inducing apoptosis is one function of oncolytic reovirus that investigators hoped could be used as future therapy, recent studies have shown that reovirus has another function that can promote antitumor activity. During their phase II trial, Noonan and colleagues discovered that treatment with Reolysin® significantly increased multiple cytokines such as

IL-10, RANTES, SDF-1, and VEGFA and concluded that reovirus exposure has some proinflammatory effects (Noonan et al., 2016).

In another investigation by Errington and colleagues, T3D reovirus was found to induce dendritic cell (DC) maturation and promote innate immunity against tumors (Errington et al., 2008). Human myeloid DCs were used in the study due to their presence in the tumor environment. DCs treated with reovirus matured and secreted inflammatory cytokines such as TNF-α, IL-6, and IL-12. These reovirus-activated DCs were then co-cultured with autologous natural killer (NK) or T cells and induced IFN-γ production in both NK and T cells indicating their activation (Errington et al., 2008). This research study provides further evidence for the immune modulating function of reovirus.

Reovirus not only helps activate innate immunity, but may also play a role in activating adaptive immunity as well. Similar to Errington's study, Prestwich and colleagues cultured DCs with T3D reovirus-infected melanoma cells and found that they induced DCs to mature. These induced DCs were then co-cultured with peripheral blood lymphocytes which then displayed lymphocyte expansion, IFN-γ production, anti-melanoma cytotoxicity, and cross-priming of CD8+ T cells against melanoma tumor associated antigen, MART-1 (Prestwich et al., 2008). Interestingly, when researchers cultured dendritic cells with melanoma cells alone they found that no induction of DC maturation or cytokine production was found.

In another study by Prestwich, researchers identified that viral lysis of tumor cells was not necessary in order to generate antitumor immunity. Researchers used melanoma cell lines B16tk (found to be susceptible to reovirus infection and killing) and B16ova (found to be resistant to reovirus infection and killing). T3D reovirus replication failed to occur when administered to B16ova cells *in vitro* or *in vivo* while reovirus-mediated oncolysis failed to occur *in vitro* only. Moreover, it was found that reovirus did not reduce tumor burden when administered to immunodeficient mice bearing B16ova cells. However, reovirus did succeed in reducing tumor burden in immunocompetent mice, supporting the idea that it may prime host immune cells in the absence of viral oncolysis (Prestwich et al., 2009).

In their investigation of the potential of reovirus as therapy for patients with chronic lymphocytic leukemia (CLL), Parrish and colleagues found that replication-competent reovirus was directly cytotoxic against CLL cells (2015). Moreover, along with direct cytotoxicity, reovirus stimulated NK cells through an IFNα-dependent mechanism, as well as enhanced antibody-dependent cellular cytotoxicity against CLL (Parrish et al., 2015). Similarly, Hall and colleagues found that T3D reovirus could also infect, replicate in, and kill acute myeloid leukemia (AML) cells of myeloblastic and monocytic origin. Moreover, reovirus was able to induce secretion of IFNα and chemokine RANTES from infected AML cells leading to activation of dendritic cells and the development of anti-leukemic responses (Hall et al. 2012).

CONCLUSION

Reovirus is a ubiquitous virus that most people are first exposed to as children when it may cause asymptomatic or benign infections resulting in cold-like symptoms or diarrhea. The development of protective antibodies likely prevents symptoms upon repeated exposures, which likely occur throughout the lifetime of the host. Reovirus has also been shown to be oncolytic, preferentially infected and killing certain types of tumor cells. Several studies have investigated whether reovirus might cause more serious diseases in humans and tested this theory by examining neutralizing antibody titers against reovirus in patients with idiopathic liver disease or primary biliary cirrhosis (Minuk et al. 1987)(Minuk, Paul, and Lee 1985). Although in both studies patients that were believed to have developed their diseases due to reovirus infections had slightly higher antibody titers when compared with healthy individuals, the difference was not significant to support reovirus as a causative agent.

Overall, after an examination of the published findings from clinical trials involving T3D reovirus (Reolysin®) treatment in solid tumors, metastatic cancers, and one blood cancer, the evidence suggests that reovirus administered as either intratumoral injection or intravenously is safe and generally well-tolerated. The studies evaluated in this investigation provide evidence to support the hypothesis that utilization of T3D reovirus as a treatment for cancers is effective and provides benefits that offset the adverse events that might occur from its

use. The maximum dose used in any study involving intratumoral injection was six injections over the course of three weeks at a dose of $1x10^{10}$ $TCID_{50}$ and was recommended for future treatments (Harrington et al. 2010). Similarly the maximum recommended dose for intravenous infusion of reovirus was $3x10^{10}$ $TCID_{50}$ for 5 consecutive days every three or four weeks.

Interestingly, Reolysin® has been found to have sufficient potential in treating a wide variety of cancers. Several studies examined in this investigation have provided evidence of reovirus infection and replication in tumors such as melanoma, myeloma, leukemia, head and neck cancer, breast cancer, sarcoma, and many others. Research is also continuously being done to examine the potential of Reolysin® treatment of cancers that have yet to be examined. For example, T3D reovirus was found to induce apoptosis in prostate cancer cells indicating its potential use in prostate cancers (Thirukkumaran et al. 2010). Another study showed cytopathic effects of T3D reovirus in peritoneal metastases of human gastric cancer (Kawaguchi et al. 2010). The potential of using reovirus to treat various cancers is also enhanced by the fact that it can be administered in multiple ways.

Both intratumoral injection and intravenous infusion are delivery methods shown to allow reovirus to reach tumors and exert its effects. However, both display advantages and disadvantages. Although NARA levels were shown to increase in all patients given intratumoral injections of reovirus, the intratumoral approach is believed to have an advantage over intravenous infusion in that it is

less likely to be affected by a NARA response because it places reovirus in direct contact with tumor cells (Harrington et al. 2010). The intratumoral approach has also shown greater efficacy in patients when compared with the intravenous approach with one complete response reported in a study by Morris and colleagues (Morris et al. 2013). These are two clear benefits of intratumoral administration of Reolysin that warrant its use in patients. However, intravenous administration allows the virus to travel through the circulatory system and encounter tumor cells throughout the body. This approach has been advantageous for blood cancers in which solid tumors are rarely available for treatment and in metastatic cancers in which lesions are found in multiple locations in the patient.

In general, both forms of administration provide benefits based on the type of cancer present, whether a primary solid tumor cancer or blood/metastatic cancer, and the main deciding factor for what treatment approach to use should be determined by the type of cancer a patient has. Primary solid tumor cancers should be treated with intratumoral injection due to the greater efficacy of treatment seen because it allows for expedited delivery to tumor, delivery of higher viral load, and avoidance of immune clearance (Kolb et al. 2015) On the other hand, blood/metastatic cancers should be treated intravenously due to the greater spread of the virus in the system to be able to encounter cancer cells that are also found in various sites in these patients.

Some major concern of intravenous infusion of reovirus are its attenuation by neutralizing antibodies in the host and ability to deliver an effective viral load to tumors (Comins et al. 2010). The future of Reolysin® as a treatment for cancer will likely be as a part of a combination therapy with immune modulators or chemotherapeutic agents. One investigation attempted to decrease the NARA response by combining reovirus treatment with cyclophosphamide, but their results demonstrated that addition of cyclophosphamide did not significantly influence host antiviral responses (Roulstone et al. 2015). However another study combining reovirus with cyclophosphamide yielded positive results in that the addition of cyclophosphamide was found to modulate neutralizing antibodies (Qiao et al. 2008). When comparing the efficacy of intravenous Reolysin® alone versus in combination therapies, the clinical trials examined in this study provide some evidence for the benefit combined therapy produces. On its own, reovirus only showed a modest efficacy when administered intravenously, however greater efficacies were seen in the combination therapies.

Interestingly, one investigation sought a new method of intravenously administering reovirus by loading it into blood mononuclear cells to avoid a NARA response (Adair et al. 2013). One extra benefit noted in the study is that this method also helped to stimulate NK cells to kill tumor targets, by inducing an innate immune-mediated response (Adair et al. 2013). Evidence has been seen for the dual ability of reovirus to directly induce tumor oncolysis and recruit innate and adaptive immune cells to target cancer cells. Unfortunately, one limitation of

the current clinical trials is that they have not fully examined this second ability of reovirus to activate host immune cells in cancer patients. Future studies and clinical trials should be conducted to examine the synergistic potential of combining reovirus with immune checkpoint inhibitors.

One research study by Carlson, Bultz, and Morris examined the quality of life and distress in patients enrolled in a phase I trial of Reolysin® (2005). The study interviewed sixteen patients with incurable metastatic cancer and found that all patients were hopeful and excited to begin the trial with 2/3 of them hoping for disease regression and 1/3 hoping for a cure (Carlson, Bultz, and Morris, 2005). Research studies and clinical trials provide hope for patients afflicted with untreatable diseases and are the key to discovering new treatments. At this time, extensive research has been done in investigating the effects of Reolysin® both alone and in combination with other therapies for patients with solid tumor cancers and metastases. However, future studies are needed in order to better evaluate the efficacy of these treatments and elucidate mechanisms that may increase their potential. Moreover, not many trials have been done investigating the efficacy of reovirus treatment in patients with blood cancers even though research studies have shown its potential. Thus future studies investigating the efficacy of Reolysin® alone and in combination with other therapies in patients with blood cancers should be done.

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