A Phase I Trial of Single-Agent Reolysin in Patients with Relapsed Multiple Myeloma

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Abstract

**Purpose:** Reolysin, a proprietary isolate of reovirus type III dearing, enters and preferentially induces apoptosis of malignant cells. RAS pathway activation has been associated with more efficient reoviral infectivity and enhanced oncolysis. Reovirus is currently in advanced solid tumor phase I-II trials; no clinical trials have been conducted in patients with hematologic malignancies.

**Experimental Design:** A phase I trial treated 12 relapsed myeloma patients at two dose levels. Reolysin was infused daily for 5 days every 28 days. Bone marrow specimens were examined by *in situ*-based hybridization (ISH) for CD138, p38, caspase-3, reoviral RNA, and capsid protein at screening and cycle 1 day 8. Junctional adhesion molecule 1 (JAM-1) and cancer upregulated gene 2 (CUG2) were evaluated in patient samples and multiple myeloma cell lines. Neutralizing anti-reovirus antibody assay was performed weekly during cycle 1.

**Results:** There were no dose-limiting toxicities, patients reached the $3 \times 10^{10}$ TCID$_{50}$ daily on days 1 to 5 dose level, and grade 3 laboratory toxicities included neutropenia, thrombocytopenia, and hyponatremia. ISH demonstrated reoviral genome confined in multiple myeloma cells. Reoviral capsid protein and caspase-3 were rarely identified within reoviral RNA-positive cells. The longest durations of stable disease were 4, 5, and 8 months.

**Conclusions:** Treatment with single-agent Reolysin was well tolerated and associated with avid reoviral RNA myeloma cell entry but only minimal intracellular reoviral protein production within multiple myeloma cells. Our data support that in multiple myeloma cells, Reolysin-induced oncolysis requires combination therapy, similar to other cancers.

**Introduction**

Reovirus serotype 3–dearing strain is a naturally occurring, ubiquitous, nonenveloped human reovirus with a genome that consists of 10 segments of double-stranded RNA (1). Community-acquired reovirus infection in humans is generally mild and limited to the upper respiratory and gastrointestinal tract. Reovirus has been shown to preferentially replicate in malignant cells with transformed RAS pathways (2–6) and mediates antitumor activity via induction of apoptosis, direct cytolysis, and activation of a tumor-directed immune response. The specificity of reovirus for cancer cells makes Reolysin, its intravenous form, an attractive drug.

Early-phase clinical trials in patients with metastatic solid tumors have shown that single-agent Reolysin is safe and well tolerated (7–9). No dose-limiting toxicities (DLT) were experienced, and the most severe side effects (grade 3 or 4) included fatigue, flu-like symptoms, neutropenia, thrombocytopenia, and hyponatremia. In these intravenous monotherapy trials, up to 45% of patients had evidence of at least stable disease, but only one patient in the 72 total enrolled achieved a partial response. Phase Ib clinical trials combined chemotherapeutic agents with Reolysin (e.g., gemcitabine, carboplatin, docetaxel, and paclitaxel) in melanoma, head and neck, prostate, ovarian, non–small cell lung, and pancreatic cancers (10–13). These trials confirmed the safety of Reolysin in combination with other agents and demonstrated an improvement in clinical response with the addition of a chemotherapeutic stressor (Supplementary Table S1). The mechanisms underlying this improved clinical efficacy are unclear; however, chemotherapy may enhance productive reoviral infection and thereby increase malignant cell apoptosis (14).

Preclinical evidence has demonstrated that Reolysin induces multiple myeloma cell oncolysis (15, 16) alone...
Translational Relevance

Reolysin is associated with clinical efficacy in patients with advanced solid tumors when combined with chemotherapy. This article describes the first use of single-agent Reolysin in hematologic malignancy; a phase I trial in relapsed multiple myeloma patients. Single-agent Reolysin was well tolerated and associated with stable disease in 50% of patients, the longest durations of which was 4, 5, and 8 months. Reoviral RNA and protein were found in bone marrow aspirates cycle 1 day 8 but not at baseline. Ample intracellular reoviral RNA was evident, but reoviral capsid protein expression and caspase-3 staining were minimal, indicating nonlytic infection. Future clinical trials combining Reolysin with a cellular stressor such as a proteasome inhibitor are needed to determine whether this combination will induce clinically relevant myeloma cell death.

and in combination with the proteasome inhibitor (PI) bortezomib, and showed that the combination synergistically upregulated endoplasmic reticulum (ER) stress-related genes and proapoptotic genes associated with ER stress (BH3 containing proapoptotic bcl-2 family members, NOXA, BIM, and PUMA). Thirukkumaran and colleagues (17) showed that the autophagosome marker, LC3-II, was greatly increased following treatment with reovirus; results suggesting that reovirus promoted autophagic cell death via ER stress-induced activation of the Akt-mTOR signaling pathway. Collectively, these studies provided preclinical justification for the use of Reolysin in patients with multiple myeloma.

Reolysin has not previously been infused into patients with relapsed hematologic malignancies. We embarked on a phase I trial of Reolysin in relapsed multiple myeloma patients to demonstrate safety and tolerability. Secondary objectives included response assessment, measurement of neutralizing anti-reoviral antibody (NARA) titers, and staining for productive reoviral infection within patient myeloma cells. In vitro and correlative analyses of multiple myeloma cell lines and patient samples were undertaken to evaluate potential markers of multiple myeloma cell sensitivity to reovirus.

Materials and Methods

Tissue culture and materials

RPML-8226 and NCI-H929 multiple myeloma cell lines were obtained from ATCC. OPM2 cells were a kind gift from Michael Kuehl (NIH, Bethesda, MD). Multiple myeloma cell lines were maintained in RPMI-1640 media supplemented with 10% FBS in a humidified incubator 37°C with 5% CO₂. Reolysin used for preclinical studies were a gift from Dr. Matt Coffey (Oncolytics Biotech Inc).

IHC

The antibody to reovirus capsid protein was a gift from Dr. Matt Coffey (Oncolytics Biotech Inc). The following antibodies were used in this study: antibody to reovirus capsid protein (compliments of Dr. Matt Coffey of Oncolytics Biotech, Inc.), caspase-3 (1:33, antigen retrieval, Abcam), p38 (1:250, antigen retrieval, Abcam), junctional adhesion molecule 1 (JAM-1), and cancer upregulating gene 2 (CUG2). The viral RNA in situ hybridization (ISH) protocol has been previously published (14, 18, 19). In brief, after digestion in protease, the tissue and reoviral RNA probes (locked nucleic acid modified 5’ digoxigenin tagged, Exiqon) were coincubated at 60°C for 5 minutes, then hybridized for 2 to 15 hours at 37°C. After a wash in 0.1 x SSC and 2% BSA at 50°C for 10 minutes, the reoviral RNA–probe complex was visualized via NBT/BCIP (Roche) due to the action of the alkaline phosphatase conjugation to antidigoxigenin antibody. Negative controls included myeloma cases not exposed to reovirus and omission of the probe; myeloma cell lines either infected or sham infected with reovirus served as additional controls.

Optimal detection of JAM-1 and CUG2 by IHC was determined using the Leica Bond Max (dilution of 1:150 with pretreatment in antigen retrieval solution 2 for 30 minutes at 95°C). Positive controls included malignant cell lines with high sensitivity to reoviral infection. The CUG2 and JAM-1 antibodies were commercially obtained from Abcam.

Detection of neutralizing anti-reovirus antibodies

Patient serum was collected at baseline and weekly for 3 weeks during the first cycle of treatment. Dilutions of patient serum were treated with a 1:1000 dilution dose of reovirus [Oncolytics; 2.53 × 10^10 50% tissue culture infectious dose (TCID_{so})/mL] known to cause 80% cell death of L929 mouse cells. The serum and virus mixture were coincubated for 2 hours to allow any antibodies in the serum to neutralize the virus before culturing with L929 cells as previously described (20). Cell survival was measured by MTT assay (ATCC) after 72 hours. Goat serum (Lampire Biological Laboratory) was used as a positive control for the NARA assay. NARA endpoint titer was expressed as the last dilution where any neutralization occurred before reovirus-only treated L929 cells (20% survival). NARA titer assay posttest trend analysis was conducted utilizing GraphPad Prism 6 software.

Patients

The Ohio State University Cancer Institutional Review Board (Columbus, OH) approved the phase I study, and informed consent was obtained from all enrolled patients (www.clinicaltrials.gov, NCT01533194). Patients with relapsed and refractory myeloma according to the International Myeloma Working Group (IMWG) diagnostic criteria for symptomatic myeloma were enrolled (21). Patients must have received prior lenalidomide and bortezomib therapy, progressed on or within 60 days of the most recent therapy, and had an Eastern Cooperative Oncology Group
The study was designed to enroll an initial cohort of 3 patients at 3 × 10^5 TCID50/day, and assuming no DLTs, an additional 9 patients would be treated at 3 × 10^6 TCID50/day. These dose levels were chosen based on tolerability in previous trials in patients with advanced solid tumors. Patients were scheduled to continue treatment for up to 12 cycles or until the time of disease progression or intolerance of therapy.

**Dose-limiting toxicity**

The Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v. 3) was used to grade toxicities. A DLT was defined as one of the following occurring during the first cycle of therapy that was determined to be possibly, probably, and definitely related to single-agent Reolysin:ANC <500/µL lasting 5 days or more or grade 4 thrombocytopenia (platelet count <25,000/µL) with bleeding; LVEF < 50% in patients that underwent screening echocardiogram or MUGA scan at screening. Adequate organ and marrow function was required with absolute neutrophil count ≥ 1,000/µL, platelet count ≥ 50,000/µL, total bilirubin < 1.5 mg/dL, and AST and ALT ≤ 5 × the institutional upper limit of normal. There was no serum creatinine requirement. Exclusion criteria included congestive heart failure with a LVEF < 50% at the time of screening.

**Study design**

This was an open-label, dose-escalating, single-center phase I trial of Reolysin (serotype 3 – replication competent adenovirus) in which Reolysin was provided by Oncolytics Biotech Inc. and distributed by the Pharmaceutical Management Branch of the National Cancer Institute (Bethesda, MD).

Reolysin was administered intravenously over 60 minutes on days 1 to 5 every 28 days. Peripheral blood was obtained for correlative analyses on days 1, 8, 15, and between days 22 and 28 of cycle 1. Females of childbearing potential underwent pregnancy testing at the time of screening and at cycle 1 day 8. Peripheral blood was obtained at screening and cycle 1 day 8. Bone marrow biopsies were obtained at screening and cycle 1 day 8. Peripheral blood was obtained for correlative analyses on days 1, 8, 15, and between days 22 and 28 of cycle 1. Females of childbearing potential underwent pregnancy testing at the time of screening.

**Results**

**Patients**

Twelve refractory patients were enrolled and demographics are summarized in Table 1. Eight patients were male, 11 were Caucasian, the median age was 61 years (range 48–77), and the median ISS stage at the time of enrollment was 2 (range 1–3). The median β-2 microglobulin was 3.2 mg/L (range 1.9–28.9) and 5 patients had a baseline creatinine < 1.0 mg/dL. All patients had received prior treatment with lenalidomide and bortezomib. The median number of prior therapies at enrollment was 5 (range 1–10) and the median number of lines of therapy was 3.5 (range 1–9) as defined by the IMWG.

**Safety and toxicities**

Three patients were treated at dose level 3 × 10^6 TCID50 and 9 patients were treated at dose level 3 × 10^7 TCID50. Treatment was well tolerated and no DLTs were experienced at either dose level. The top ten worst grade adverse events are listed in Supplementary Fig. S1. Grade 3 toxicities were minimal and included 3 patients with neutropenia, 2 with leukopenia, and one each with thrombocytopenia and hypophosphatemia. Grade 2 toxicities included leukopenia, anemia, neutropenia, thrombocytopenia, and myalgias. Other grade 1 toxicities included increased aspartate aminotransferase and alkaline phosphatase, lymphopenia, and nausea. All grade 2 and 3 toxicities occurred in patients treated at dose level 2. Five different patients had evidence of grade 2 and/or 3 hematologic toxicities reported as possibly or probably related to Reolysin. One patient had pancytopenia on day 4 of cycle 1. Resolution was evident by day 11 of cycle 1, and this patient had an associated increase of serum lambda-free light chain from 1,435 to 3,000 mg/L over the course of cycle 1. A second patient had a decrease of the neutrophil and total white blood cell count evident on day 8 of cycle 1. These adverse events were associated with an initial 10% decrease of the m-protein, but the patient rapidly progressed and received only one cycle of therapy. A third patient had evidence of decreased neutrophils, total white blood cell count, and platelets on day 4 of cycle 1. All cytopneas resolved by day 23 of cycle 1 and were associated with a minimal decrease of the serum lambda-free light chains during cycle 1. Ultimately, this patient received a total of two cycles of treatment. The fourth patient had stable disease over the course of five cycles of treatment and...
did not have evidence of cytopenias during cycle 1, but developed grade 3 decreases of the neutrophil and total white blood cell count during cycle 2. These toxicities were evident on day 4 of cycle 2, and resolved by the start of cycle 3 (see Supplementary Table S2).

**IHC**

IHC analysis of sensitive (RPMI-8226), intermediately resistant (H929), and resistant (OPM2) multiple myeloma cell lines indicated two primary findings: (i) reoviral RNA was present in all cell lines and increased with higher multiplicities of infection (MOI) and (ii) viral capsid protein was evident in all cell lines, but to a greater degree in resistant cell lines. Of the evaluable patient samples, reoviral infection (22). Of the evaluable patient samples, reoviral RNA localized to myeloma cells and increased with higher MOI (Fig. 1A–C). Further, these findings indicate that resistance to reovirus may be defined by a lack of viral genome and/or proliferation in infected cells.

Seven patients had adequate tissue samples for both pre- and posttreatment laboratory evaluation (Table 2). Reoviral RNA was found in post-Reolysin bone marrow biopsies in all 7 evaluable patients, and viral protein was found in 5 of the 6 evaluable patient samples. Results confirmed reoviral cell entry as evidenced by positive viral RNA staining in 20% to 100% of myeloma cells (Fig. 1D). There was no association ($P = 0.22$) between the percentage of myeloma cells positive for viral RNA and the number of cycles of therapy patients received. Reoviral RNA localized to myeloma cells (Fig. 2A and B). Of the evaluable patient samples, reoviral capsid protein expression and caspase-3 staining were minimal, indicating nonproductive infection and limited induction of apoptosis (table 2). Importantly, caspase-3 expression paralleled reoviral capsid protein expression. Expression of p38 was used as an indicator of RAS pathway activation and multiple myeloma cell permissiveness to reoviral infection (22). Of the evaluable patient samples, p38 expression before treatment was evident in a mean of 72% (range 19%–99%) of myeloma cells (defined by CD138 coexpression), and was 94% and 99% in patients experiencing stable disease for 8 and 4 cycles of treatment, respectively.

### Table 1. Patient demographics

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>ISS stage at screening</th>
<th>B2M CR</th>
<th>Revlimid exposed/ refractory</th>
<th>Velcade exposed/ refractory</th>
<th>Number of prior therapies</th>
<th>Number of prior lines of therapy</th>
<th>Cytogenetics at screening</th>
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<td>50 F</td>
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<td>0.9 Y/N</td>
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<td>ND</td>
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<tr>
<td></td>
<td>B</td>
<td>77 M</td>
<td>C</td>
<td>2</td>
<td>3.6</td>
<td>1.1 Y/Y</td>
<td>Y/N</td>
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<td>7</td>
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<tr>
<td></td>
<td>C</td>
<td>48 M</td>
<td>C</td>
<td>2</td>
<td>2.6</td>
<td>1.1 Y/N</td>
<td>Y/N</td>
<td>5</td>
<td>2</td>
<td>13q14–, 5q+, 19p+, 11q22 tetrasomy, 1q21+</td>
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<td>C</td>
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<td>8.1</td>
<td>1.7 Y/N</td>
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<td>7</td>
<td>6</td>
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<tr>
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<td>Y/N</td>
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<td>1</td>
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<td>9</td>
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<td></td>
<td>H</td>
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<td>0.3 Y/Y</td>
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<td>3</td>
<td>1</td>
<td>Normal</td>
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<td></td>
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<td>76 M</td>
<td>C</td>
<td>3</td>
<td>9.8</td>
<td>4.3 Y/N</td>
<td>Y/N</td>
<td>5</td>
<td>3</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>48 M</td>
<td>C</td>
<td>1</td>
<td>1.9</td>
<td>0.8 Y/Y</td>
<td>Y/N</td>
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<td>5q+, 19p+</td>
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<td>Y/N</td>
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<td>4</td>
<td>13q14–, 5q+, 19p+, 11q22+, 12p–, 1q21+, 1q23+, 1p36+</td>
</tr>
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<td>62 M</td>
<td>AA</td>
<td>1</td>
<td>2.4</td>
<td>1.2 Y/Y</td>
<td>Y/Y</td>
<td>5</td>
<td>3</td>
<td>19p+, 11q22+, 12p–, 1q21+, 1q23+, 1p36+</td>
</tr>
</tbody>
</table>

NOTE: Patient demographics of all patients enrolled on the phase I single-agent Reolysin trial. Patient factors included age, gender, and race; ISS stage, β-2 microglobulin, cytogenetics, and serum creatinine at the time of screening; assessment of prior Revlimid and/or Velcade exposure, and number of prior therapies and lines of therapy. Abbreviations: B2M, β2 microglobulin at screening; Cr, serum creatinine at screening; ISS, International Staging System; F, female; M, male; Y, yes; N, no; C, Caucasian; AA, African American; NA, not applicable; ND, not determined; DL1, dose level 1 (3 × 10¹⁰ TCID₅₀/d); DL2, dose level 2 (3 × 10¹⁵ TCID₅₀/d).
JAM-1 mediates clathrin-dependent viral internalization and CUG2 has been shown to inhibit the phosphorylation of PKR, so we analyzed their respective roles in reoviral permissiveness in multiple myeloma cells. IHC analysis of JAM-1 and CUG2 in sensitive (RPMI-8226), intermediately resistant (H929), and resistant (OPM2) multiple myeloma cell lines indicated that JAM-1 was expressed in sensitive cell lines only, whereas CUG2 was expressed in all cell lines (Fig. 3A). CUG2 expression was lowest in resistant cell lines and highest in sensitive cell lines. These findings highlighted the possibility that JAM-1 and CUG2 may be markers of reovirus sensitivity in multiple myeloma cells.

Patient samples were analyzed for coexpression of JAM-1/CD138 and CUG2/CD138. Low-null JAM-1 expression was evident in all evaluable patient samples, suggesting that decreased tropism of the virus for multiple myeloma cells is associated with decreased viral efficiency in vivo. CUG2 was evident in two of the evaluable patient samples. One patient had evidence of CUG2 expression in the majority of multiple myeloma cells pre- and posttreatment (Fig. 3B). This patient progressed after two cycles of treatment and had relatively high p38 expression (98%) on pretreatment sample analysis. Analysis of pre- and posttreatment samples from the patient experiencing the longest duration of stable disease indicated only scattered CUG2 expression. Pretreatment analysis revealed that this patient also had relatively high p38 expression (94%).

**NARA response**

Evaluation of the anti-Reolysin immunologic response revealed that all patients had NARA titers in their serum posttreatment. These data indicate that each patient developed neutralizing antibodies against reovirus (Fig. 4). Trend analysis indicated a significant increase \( P = 0.001 \).
NARA titers from pretreatment to week 3. Evaluating the 3 patients with the longest duration of stable disease (4, 5, and 8 months) suggested higher week 1 and 2 endpoint titers in the patient receiving eight cycles of treatment. These data suggest that NARA antibodies may be indicative of a beneficial antitumor immune response mediated by the activation of a cross-reactive T-cell population.

**Response assessment**

All twelve enrolled patients were eligible for response assessment. A total of 6 patients (50%) received more than one cycle of treatment, the median cycles of therapy was 1.5, and the best response was stable disease. One patient received $3 \times 10^6$ TCID$_{50}$ and had minimal evidence of apoptosis (caspase-3) on the posttreatment bone marrow biopsy. This patient completed eight cycles of treatment and experienced stable disease for 8 months. Significantly, this patient was also noted to have a progressive increase of NARA and a concomitant decrease in the monoclonal protein during cycle 1. Two other patients receiving $3 \times 10^{10}$ TCID$_{50}$ had stable disease and were each treated for four and five cycles, both had stable monoclonal proteins.

### Table 2. IHC

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Reovirus RNA</th>
<th>Reovirus protein</th>
<th>p38</th>
<th>Caspase-3</th>
<th>Cycle of treatment</th>
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<tbody>
<tr>
<td>A</td>
<td>36%</td>
<td>9%</td>
<td>94%</td>
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</tr>
<tr>
<td>B</td>
<td>100%</td>
<td>14%</td>
<td>78%</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>100%</td>
<td>11%</td>
<td>98%</td>
<td>0.3%</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
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<td>3%</td>
<td>19%</td>
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<td>I</td>
<td>20%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5</td>
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</table>

**NOTE:** IHC analysis of 7 evaluable patient samples; coexpression analyses included CD138$^+$, reoviral RNA, capsid protein, p38, and caspase-3.

**Abbreviations:** Reovirus RNA, reoviral RNA/CD138$^+$ cells posttreatment; Reovirus protein, reoviral protein/CD138$^+$ cells posttreatment; p38/CD138$^+$, p38 pretreatment cells; caspase-3, caspase-3 posttreatment.

![Figure 2](http://example.com)  
Coexpression analyses with IHC. Coexpression analysis for reoviral RNA (locked nuclear acid ISH) and CD138 were completed on pre- (A) and posttreatment (B) patient samples. The Nuance system was used to convert reoviral RNA to blue, CD138 to red, and coexpression is yellow. Reoviral RNA-positive cells are primarily CD138$^+$ cells indicating that reovirus selectively enters myeloma cells.
during cycle 1, but caspase-3 staining was not determined on posttreatment bone marrow samples. During cycle 1, a total of 5 patients had decreased myeloma proteins (range 6%–22%, mean 14%), 3 had minimal increases (6, 7, and 12%), and the remaining 4 had evidence of grossly progressive disease.
1010 TCID50/day) were well tolerated. No DLTs were experienced and grade 3 toxicities were limited to neutropenia, leukopenia, thrombocytopenia, and hypophosphatemia.

109 TCID50/day dose, had evidence of reoviral capsid protein suggested resistance to active viral infectivity, and apoptosis-mediated viral release (26). We evaluated p38 MAPK as a marker of RAS-pathway activation (22), potentially via EGFR, N- and/or K-RAS mutations. In multiple myeloma, the proportion of RAS mutations increases with disease progression, and 46% to 81% of patients with relapsed disease have been found to have N- or K-RAS mutations (24, 25). Recently, a study evaluating the prevalence of RAS mutations in multiple myeloma patient samples from 133 patients demonstrated N- and K-RAS mutations in similar proportions, and patients with N-RAS–mutated tumors, in comparison with those with wild-type tumors, were less sensitive to bortezomib (25). The mechanisms underlying this decreased bortezomib sensitivity of N-RAS–mutated tumors require further investigation. Regardless, these findings are instrumental in confirming the equivocal prevalence of N- and K-RAS mutations in multiple myeloma.

The role of a constitutively activated RAS pathway in multiple myeloma patients treated with Reolysin was explored in our trial. Numerous studies suggest that reovirus inhibits double-stranded RNA-activated protein kinase (PKR) autophosphorylation in RAS-mutated cells, an effect that permits viral replication to occur (2–6). Others suggest that RAS transformation enhances numerous steps in the viral life cycle, including proteolytic disassembly, degree of infectivity, and apoptosis-mediated viral release (26). We evaluated p38 MAPK as a marker of RAS-pathway activation in our relapsed population (22) and found no obvious association between p38, length of disease stability, or the amount of viral protein present in malignant cells (Table 2).

Of the myriad of factors that could mediate the equivocal prevalence of N- and K-RAS mutations in multiple myeloma, we have successfully shown that Reolysin is well tolerated. No DLTs were experienced and grade 3 toxicities were limited to neutropenia, leukopenia, thrombocytopenia, and hypophosphatemia. Single-agent Reolysin was not associated with any objective responses in relapsed and refractory multiple myeloma patients. Six of the 12 (50%) patients in this study received more than one cycle of therapy and 3 patients received 4, 5, and 8 cycles of treatment. The longest duration of stable disease was 8 months. This patient was treated with the 3 x 10^9 TCID50/day dose, had evidence of reoviral capsid protein expression in CD138cells, apoptosis on the posttreatment bone marrow biopsy (Table 2), and a 13% decrease in the monoclonal protein during cycle 1.

Preclinical models suggest that reoviral cell entry is initiated by the engagement of the outer viral capsid filamentous attachment protein σ1 with JAM-1 and a cell-surface carbohydrate. Once attached, β1-integrin–mediated viral internalization occurs via clathrin-dependent endocytosis. Following internalization, acid-dependent cysteine proteases in the endocytic compartment remove the outer capsid protein σ3, exposing a viral membrane-penetration protein that is responsible for piercing the endosomal membrane and delivering transcriptionally active virus into the cytoplasm (23).

Whether these fundamental principles accurately define the in vivo mechanisms of cell entry and viral proliferation in multiple myeloma cells is unknown. To elucidate the importance of JAM-1, we conducted in vitro analyses of sensitive, intermediately resistant, and resistant cell lines, and correlative analyses of patient samples. In vitro analyses confirmed that JAM-1 expression was positively associated with multiple myeloma cell sensitivity (Fig. 3A). However, IHC analysis of patient samples revealed that JAM-1 was not expressed on myeloma cells. Considering that viral RNA and not protein was evident in patient samples, we propose that reovirus may have passively, albeit selectively, entered the myeloma cells by bypassing JAM-1. This hypothesis assumes that reovirus binding to the sialic acid and JAM-1 receptors is necessary for viral uncoating and subsequent replication. Future strategies using therapeutics that upregulate JAM-1 will likely elucidate whether active clathrin-dependent reoviral cell entry is necessary for viral proliferation, viral-mediated apoptosis, and subsequent clinical benefit.

Reovirus has been shown to preferentially replicate and induce apoptosis in cells with a constitutively activated RAS pathway (22), potentially via EGFR, N- and/or K-RAS mutations. Single-agent Reolysin was not associated with any objective responses in relapsed and refractory multiple myeloma patients. Six of the 12 (50%) patients in this study received more than one cycle of therapy and 3 patients received 4, 5, and 8 cycles of treatment. The longest duration of stable disease was 8 months. This patient was treated with the 3 x 10^9 TCID50/day dose, had evidence of reoviral capsid protein expression in CD138 cells, apoptosis on the posttreatment bone marrow biopsy (Table 2), and a 13% decrease in the monoclonal protein during cycle 1.

We propose that the lack of objective clinical response in our population resulted from a combination of potential factors, including (i) an inability to overcome inherent viral resistance for productive cytolysis, (ii) limited production of a viral-mediated antitumor immune response, and/or (iii) inadequate viral dosing.

As seen in our patient population (Fig. 1D), and supported by our in vitro analyses of sensitive, intermediate resistant, and resistant multiple myeloma cell lines (Fig. 1A–C), the presence of intracellular viral genome but not reoviral capsid protein suggested resistance to active viral proliferation. Of the myriad of factors that could mediate this resistance, we evaluated the roles of cell entry and RAS pathway activation.

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Cancer upregulated gene 2 (CUG2) is a novel oncogene that is overexpressed in numerous malignancies. Like RAS mutations, CUG2 expression is associated with inactivation of PKR and activation of the RAS/p38 MAPK signaling pathway (27). We evaluated the role of CUG2 in vitro and in patient samples. Preclinical analysis of sensitive, intermediately resistant, and resistant multiple myeloma and melanoma cell lines indicated that CUG2 was more highly expressed in sensitive cell lines (Fig. 3A). In these models, increased viral load and concomitant exposure to chemotherapy increased CUG2 expression, viral proliferation, and apoptosis (Fig. 3C). These results suggest that in vitro, CUG2 expression enhances reovirus sensitivity and cytosis. Analysis of patient samples revealed that 2 of the 7 evaluable patients had evidence of CUG2 expression. One patient had significant pre- and posttreatment CUG2 expression in multiple myeloma cells (Fig. 3B), viral genome in 100% of cells, elevated pretreatment p38 expression (98%), minimal reovirus capsid protein and apoptotic activity, and received two cycles of treatment (patient C). In the other patient, CUG2 expression was scattered, 36% of cells contained viral genome, pretreatment p38 expression was high (94%), reoviral capsid protein, and apoptosis were minimal, and the patient received eight cycles of treatment (patient A). Collectively, these results suggest that CUG2 expression may be an indicator of RAS/p38 pathway activation and entry of the virus into multiple myeloma cells, but not productive infection or clinical response.

Natural killer (NK) and T-cell-mediated killing has a prominent role in reovirus-mediated antimultiple myeloma activity (28, 29). Dendritic cells (DC) have the ability to directly respond to reoviral RNA via pattern recognition receptors (PRR) in a viral replication independent manner (30). DC activation by reovirus induces the upregulation of numerous proinflammatory cytokines including IFNs that function, in part, to increase NK cell activation and recruitment. Extensive cross-talk between DCs and NK cells via IFN subsequently acts to generate a tumor-reactive cytotoxic T-cell response; one that is likely strengthened by production of viral- and NK cell-mediated tumor-associated antigens (28). Numerous patients in our trial had experienced stable disease that was not associated with significant viral proliferation or apoptosis. This finding suggests that disease stability may have resulted from immune-mediated cytoxicity.

Virus clearance may be mediated by NARA, but the degree to which its activity limits reovirus-mediated antimultiple myeloma activity is unknown. Evidence from the REO 005 trial indicates that antitumor activity persisted despite a 250-fold median increase in NARA titers (20), likely because the virus “hitchhikes” on peripheral blood mononuclear cells and platelets (31). In our trial, a significant increase in NARA titers was evident over the first cycle of treatment (Fig. 4). Though our findings are not conclusive, it is unlikely that NARA is able to clear a significant proportion of infused reovirus to preclude antitumor activity in patients with myeloma, and in fact, may be instrumental in activating a beneficial antitumor immune response.

Use of viral oncolytics as cytolytic therapy is gaining momentum. Recently, Rüssel and colleagues reported on 2 patients with refractory multiple myeloma that were treated with 10^{11} TCID₅₀ MV-NIS, an engineered measles virus (32). Initial infusion resulted in hypotension, tachycardia, and high fever in both patients, but each responded to treatment and one achieved complete remission lasting for over 9 months. In fact, as this was a dose-escalation trial starting at 10^{6} TCID₅₀, no objective responses were noted until the administration of 10^{11} TCID₅₀, results suggesting that treatment at or near the MTD is necessary to achieve objective responses. Reolysin as monotherapy or combined with chemotherapy has not reached the MTD with individual doses up to 3 × 10^{10} TCID₅₀ for 5 consecutive days with cycles repeated every 21 to 28 days (total cycle dose of 1.5 × 10^{15}). Currently, the NCIC Clinical Trials Group is conducting a 4-arm phase II trial (www.clinicaltrials.gov - NCT01708993) and this trial is the first to evaluate higher individual doses of Reolysin at 4.5 × 10^{10} TCID₅₀, days 1 to 3 every 3 weeks, total cycle dose 1.35 × 10^{11} TCID₅₀. Considering the dose–response relationship evident with other viral oncolytics (33–36), higher individual doses of Reolysin may be necessary to induce clinical benefit in patients with multiple myeloma.

Clearly, further study will be necessary to define a role for Reolysin treatment in multiple myeloma. Our trial, however, shows that Reolysin is well tolerated and associated with disease stability, though the mechanisms underlying this activity remain unclear. Importantly, our study lays the groundwork for future clinical trials that will further examine the role for Reolysin in treatment refractory multiple myeloma patients, either as a single agent or in combination.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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