The Clinical Oncolytic Reovirus Formulation Reolysin Synergistically Augments the Anti-Leukemic Activity of Azacitidine

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Abstract

Despite the recent development of new agents for acute myeloid leukemia (AML) therapy, novel approaches are still needed for patients that do not benefit sufficiently from existing regimens. Reolysin (Pelareorep) is a proprietary clinical formulation of the naturally occurring oncolytic reovirus that is non-pathogenic and preferentially replicates in cancer cells, but not in normal tissue. Although Reolysin has been investigated in over 30 adult clinical trials and is very well tolerated when given alone and in combination with chemotherapy, it has never been evaluated for AML therapy. Our major goal was to determine the efficacy and mechanism of action of Reolysin alone and in combination with azacitidine in AML models and primary patient specimens.

Reolysin exhibited dose-dependent effects against AML cells with respect to the reduction in leukemia cell viability and induction of apoptosis in all 8 cell lines evaluated. The combination of Reolysin with azacitidine yielded synergistic benefit across all cell lines that was dramatically superior to single agent treatments (p<0.001). The benefit of combination treatment was confirmed in primary AML specimens (N=14 to date). RNASeq-based transcriptome and gene ontology (GO) analyses of the pharmacodynamic effects of each agent and the combination revealed that the Reolysin and azacitidine combination potently altered multiple genes in the immune response pathway (p<0.001). qRT-PCR analyses and ELISA assays confirmed the broad immunomodulatory effects of the Reolysin plus azacitidine combination. Immune priming strategies that can turn "immune cold" cancers to "immune hot" may significantly augment the benefit of many therapeutic approaches. *Basic leucine zipper transcription factor ATF-like 2* (*BATF2*) is a key tumor suppressor that is absent in AML and certain other malignancies. Its deficiency promotes immune escape of cancer cells. *BATF2* exhibits anticancer activity through upregulation of interleukin-12 and the activation of CD8+ T cells and therefore represents a novel new target in anticancer treatment with immune checkpoint inhibitors. Our transcriptome analyses identified *BATF2* as one of the most significantly upregulated genes in AML cells following treatment with the Reolysin plus azacitidine combination (p<0.0001). We hypothesize that *BATF2* induction was a key driver of the anti-leukemic effects of combination treatment.

We next evaluated the effects of Reolysin and azacitidine in the MOLM-13 AML mouse xenograft model. Reolysin (5 x 10^8 TCID50 IV 1x per week), azacitidine (5 mg/kg SC 2x per week), and the combination were administered to mice bearing MOLM-13 FLT3-ITD+ xenografts for 19 days to assess the efficacy and tolerability of each treatment. Combination treatment was very well tolerated and antagonized disease progression significantly more effectively than either monotherapy (p<0.01). Additional *in vivo* studies more rigorously assessing the immunomodulatory effects of Reolysin and azacitidine in immune competent mice are underway.

Our collective data demonstrate that Reolysin is a safe and well tolerated agent with potent immunomodulatory effects that synergistically augments the anti-AML effects of azacitidine. A Phase I investigator-initiated clinical trial further investigating the safety and preliminary efficacy of this combination in patients with AML is currently being planned.

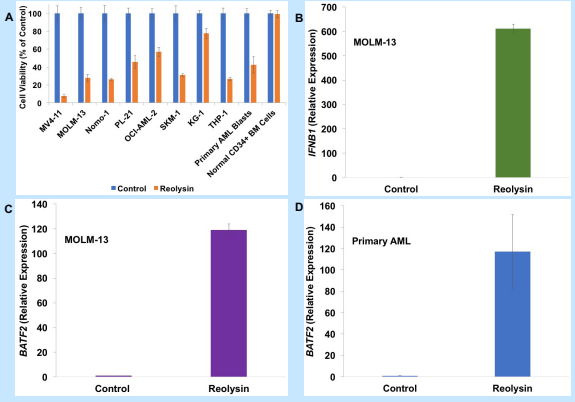
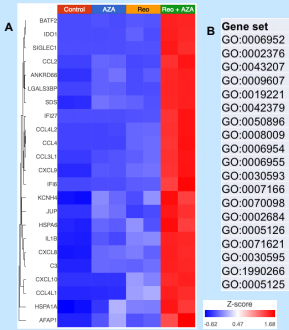


Figure 1. Reolysin selectively decreases the viability of AML cell lines and primary AML cells and induces *BATF2* expression. (A) Reolysin reduces AML cell viability. A panel of AML cell lines, primary AML blasts (n = 14), and normal CD34+ BM cells were treated with 30 PFU/cell Reolysin for 72 h and cell viability was measured by MTT assay. Mean ± SD, n = 3. (B) Reolysin induces *IFNB1* expression. MOLM-13 AML cells were treated with 30 PFU/cell Reolysin for 48 hours and *IFNB1* levels were detected by qRT-PCR. Mean ± SD, n = 3. (C-D) Reolysin upregulates *BATF2* in MOLM-13 AML cells and primary AML blasts. Cells were treated with 30 PFU/cell Reolysin for 48 hours. *BATF2* expression was determined by qRT-PCR. Mean ± SD, n = 3.

Results



B

Gene set	Description	Enrichment score	P-value
GO:0006952	defense response	54.2876	2.65E-24
GO:0002376	immune system process	49.1826	4.37E-22
GO:0043207	response to external biotic stimulus	38.3222	2.27E-17
GO:0009607	response to biotic stimulus	37.3572	5.97E-17
GO:0019221	cytokine-mediated signaling pathway	37.1126	7.62E-17
GO:0042379	chemokine receptor binding	35.2572	4.88E-16
GO:0050896	response to stimulus	34.3412	1.22E-15
GO:0008009	chemokine activity	34.1854	1.42E-15
GO:0006954	inflammatory response	33.9184	1.86E-15
GO:0006955	immune response	33.5161	2.78E-15
GO:0030593	neutrophil chemotaxis	32.4386	8.17E-15
GO:0007166	cell surface receptor signaling	32.2325	1.00E-14
GO:0070098	chemokine-mediated signaling	32.1379	1.10E-14
GO:0002684	positive regulation of immune system	31.5008	2.09E-14
GO:0005126	cytokine receptor binding	31.0581	3.25E-14
GO:0071621	granulocyte chemotaxis	30.761	4.37E-14
GO:0030595	leukocyte chemotaxis	30.5207	5.56E-14
GO:1990266	neutrophil migration	30.5079	5.63E-14
GO:0005125	cytokine activity	30.2408	7.36E-14

Figure 2. Transcriptome analysis of Reolysin in combination with azacitidine. (A) MOLM-13 AML cells were treated with 1 μM azacitidine, 10 PFU/cell Reolysin, or the combination for 48 hours. RNA sequencing analysis identified genes that were increased by at least 5 fold following combination treatment. One of the most upregulated genes in the combination treated cells was *BATF2*, p < 0.05. (B) Gene ontology analysis of MOLM-13 cells following treatment with the Reolysin and azacitidine combination. Gene ontology analysis identified an enrichment of pathways associated with immune and stress responses. Enrichment scores of 30 or greater are shown.

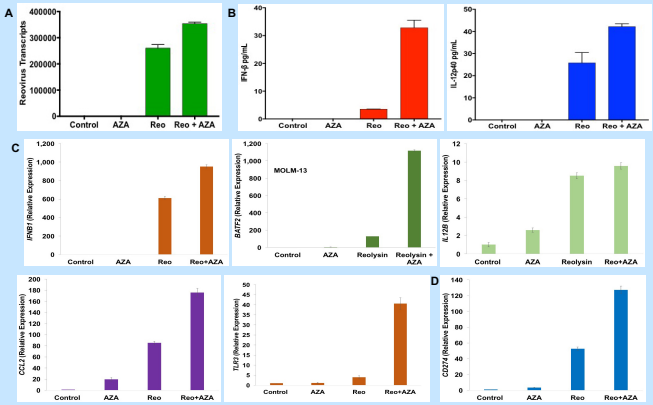


Figure 3. Azacitidine augments the effects of Reolysin. (A) Treatment with azacitidine enhances reovirus levels in MOLM-13 AML cells. MOLM-13 cells were treated with 10 PFU/cell Reolysin, 1 μM azacitidine, or the combination for 48 hours. Reovirus transcripts were determined by qRT-PCR. Mean ± SD, n = 3. (B) Azacitidine enhances the levels of IFN-β and IL-12 secretion. MOLM-13 cells were treated with 10 PFU/cell Reolysin, 1 μM azacitidine, or the combination for 48 hours. Levels of secreted IFN-β and IL-12 were measured by ELISA. Mean ± SD, n = 3. (C) Azacitidine augments the effects of Reolysin *IFNB1*, *BATF2*, *IL12B*, *CCL2*, and *TLR3*. MOLM-13 cells treated with 10 PFU/cell Reolysin, 1 μM azacitidine, or the combination for 48 hours. Gene expression was determined by qRT-PCR. Mean ± SD, n = 3. (D) Azacitidine and Reolysin increase PD-L1 levels. MOLM-13 cells were treated with 10 PFU/cell Reolysin, 1 μM azacitidine, or the combination for 48 hours. *CD274* (PD-L1) expression was determined by qRT-PCR. Mean ± SD, n = 3.

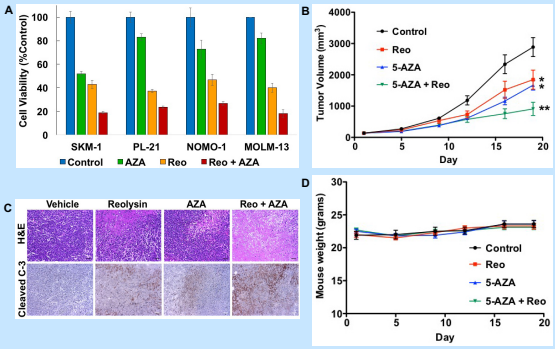


Figure 4. Reolysin augments the anticancer activity of azacitidine. (A) Reolysin enhances the *in vitro* anti-AML activity of azacitidine. AML cell lines were treated with 10 PFU/cell Reolysin (MOLM-13 and SKM-1) or 30 PFU/cell Reolysin (PL-21 and NOMO-1), 1 μM azacitidine, and the combination for 72 hours. Cell viability was determined by MTT assay. Mean ± SD, n = 3. (B) Reolysin augments the anti-AML activity of azacitidine *in vivo*. MOLM-13 tumors were established in nude mice. Mice were treated with 5 x 10^8 TCID50 Reolysin IV once a week, 5 mg/kg azacitidine SC twice a week, or the combination of both agents for 19 days. Tumor volume was measured biweekly. Mean ± SEM, n = 10. *Indicates a significant difference from Vehicle Control or ** either single agent treatment group. (C) Immunohistochemistry of tumor samples. Tumors were collected at the end and stained with H&E. Cleaved caspase-3 was measured by immunohistochemistry. (D) The combination of Reolysin and azacitidine is very well tolerated in mice. Animals were treated with Reolysin and azacitidine as described in (B). Mouse weight was measured twice a week. Mean ± SD, n = 10.

Summary

- **Reolysin selectively reduces the viability of AML cell lines and primary AML blasts.**
- **Reolysin treatment induces IFN-β and the transcription factor *BATF2*.**
- **Treatment with Reolysin enhances the anti-AML activity of azacitidine.**
- **Transcriptome analysis of MOLM-13 AML cells treated with Reolysin, azacitidine, and the combination reveals an increase in genes associated with immune response including *BATF2*.**
- **Treatment with the combination of Reolysin and azacitidine promotes increased levels of reovirus and a dramatic further induction of *BATF2*.**
- **The combination of Reolysin and azacitidine significantly diminishes AML cell viability *in vitro* compared to either single agent treatment.**
- **Reolysin and azacitidine significantly decrease tumor burden in the MOLM-13 AML xenograft mouse model.**
- **Reolysin and azacitidine increase the expression of PD-L1 suggesting that this treatment may sensitize AML cells to anti-PD-1/PD-L1 therapy.**
- **Additional *in vivo* studies more rigorously assessing the immunomodulatory effects of Reolysin and azacitidine in immune competent mice are ongoing.**
- **Taken together, our data demonstrate that Reolysin exhibits significant activity in AML models and enhances the efficacy of azacitidine.**