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^{225}Ac-CD33 Radioimmunotherapy Potently Increases the Sensitivity of Resistant Acute Myeloid Leukemia Lines to the Bcl-2 Inhibitor Venetoclax by Mediating a Reduction in Cellular Mcl-1 Levels

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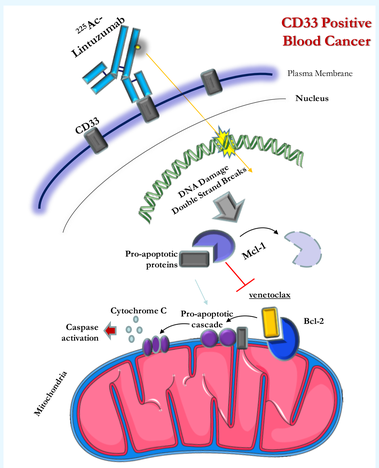
Abstract

Acute myeloid leukemia (AML) is a complex hematological disease often occurring in older patients. While a number of new targeted therapies have been recently approved, patient outcomes remain poor. In the US, about 19,520 new cases AML occur annually and approximately 10,670 will die from the disease. Venetoclax is a promising new targeted therapy that is under regulatory review in the US for use in combination with a hypomethylating agent (HMA) or with low-dose cytarabine (LDAC) for the treatment of newly diagnosed patients with acute myeloid leukemia (AML) who are ineligible for intensive chemotherapy. Recently, several studies have demonstrated that increased expression of Mcl-1 is a mediator of resistance to venetoclax in AML and other hematologic malignancies. Indeed, significant up-regulation of Mcl-1 has been reported in patients with relapsed/refractory AML and venetoclax treatment itself has been shown to increase Mcl-1 levels tumor cell lines. ^{225}Ac-lintuzumab is a clinical stage radioimmunotherapy targeting CD33 that has shown evidence of single agent activity in relapsed/refractory AML. ^{225}Ac-lintuzumab enables delivery of the potent alpha emitting warhead 225-actinium (T1/2 = 10 days; high linear energy transfer = 6.83 MeV; and short path length = 40-100 μm or about 3-5 cell diameters) directly to tumor cells. This elicits DNA cluster breaks and double strand breaks to kill within a short radius, thereby limiting damage to normal tissue. In this study, we demonstrate that ^{225}Ac-lintuzumab is capable of dramatically enhancing the potency of venetoclax when used in combination in both venetoclax sensitive and resistant AML cell lines. AML lines U937 and OCI-AML3 are Mcl-1 positive AML lines which are highly resistant to venetoclax (IC50 > 1 μM). While treatment of these lines with single agent venetoclax at 500 nM was ineffective at suppressing tumor cell growth in either model, the combination of 40 nCi ^{225}Ac-lintuzumab plus 500 nM venetoclax induced a statistically significant increase tumor cell killing in vitro. While ^{225}Ac-lintuzumab can directly effect tumor cell killing by multiple mechanisms, we investigated the potential for ^{225}Ac-lintuzumab-directed DNA damage to suppress Mcl-1 levels as a potential mechanism of enhancing the potency of venetoclax in these models. Cell lines were incubated with titrations of ^{225}Ac-lintuzumab prior to analysis of Mcl-1 protein by Western blot. In both cell lines, treatment with ^{225}Ac-lintuzumab lead to a significant depletion of Mcl-1 levels in cells. This supports a mechanistic basis of Mcl-1 suppression when ^{225}Ac-lintuzumab is used in combination with venetoclax to increase the potency of resistant AML cells to the Bcl-2 inhibitor. Results of in vivo tumor xenograft studies will also be presented.

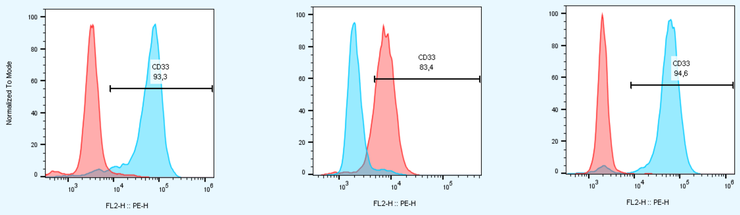
Figure 1: Proposed Mechanism of Action



- Anti-apoptotic protein Mcl-1 is a mediator of resistance to venetoclax[1]
- Mcl-1 is up-regulated in refractory AML[2]
- Anti-CD33 radio-conjugate ^{225}Ac-Lintuzumab is a potent inducer of DNA double strand breaks leading to tumor cell death in AML[3]
- DNA damage caused by radiation results in a reduction of Mcl-1 levels[4]
- ^{225}Ac-Lintuzumab mediated reduction in Mcl-1 in AML will sensitize potentially resistant cells to venetoclax
- Combination treatment of ^{225}Ac-Lintuzumab radiotherapy induced DNA damage with venetoclax will act synergistically to increase cell death in AML cell lines and primary patient samples

[1]Niu et al. 2016 Clinical Cancer Research; [2]Kaufmann et al. 1998 Blood; [3]McDevitt et al. 2001 Science; [4]Li et al. 2016 Apoptosis; [5]Figure made in part with Servier Medical Art

Figure 2: Quantification of CD33 Expression on AML Cell Lines



Cell Line	MFI	Venetoclax Sensitivity
U937	64,000	Resistant
OCI-AML3	8,099	Resistant
MOLM-13	57,000	Sensitive

Figure 2: Surface CD33 antigen levels for each AML line was determined by flow cytometry (Mean Fluorescence Intensity, MFI) using the QiFiKit (Dako) according to manufacturer's specifications.

Conflict-of-Interest Disclosure: D.L.L. and E.M.G. have equity ownership in and are employed by Actinium Pharmaceuticals, Inc. E.D., and W.D. receive research funding from Actinium Pharmaceuticals, Inc.; R.G. and K.A. have no disclosures.
*R.G. and E.M.G. are co-first authors

Figure 3: Single Agent Cytotoxicity of Venetoclax or ^{225}Ac-Lintuzumab

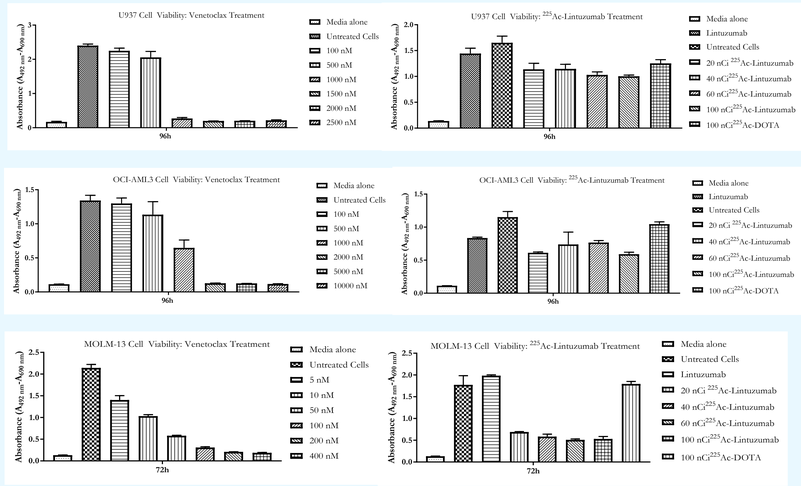


Figure 3: The AML cell lines were treated individually with a dilution series of venetoclax (A) or ^{225}Ac-Lintuzumab (B) and cell viability was measured using the XTT assay (Sigma). Cells were treated individually with venetoclax for the indicated number of hours and then cell viability was measured. For ^{225}Ac-Lintuzumab treated cells, cells were incubated with the indicated amount of antibody for 1 hr, washed, and then cultured for the indicated time period before performing the XTT assay. While venetoclax resistant cell lines U937 and OCI-AML3 were minimally affected by venetoclax, MOLM-13 cells were highly sensitive to venetoclax treatment. Likewise, treatment of U937 and OCI AML3 cells with ^{225}Ac-Lintuzumab alone did not substantially affect cell viability, the viability of MOLM-13 cells was affected at concentrations as low as 40 nCi ^{225}Ac-Lintuzumab.

Figure 4: Combination Treatment of AML Cell Lines with Venetoclax and ^{225}Ac-Lintuzumab Results in Enhanced Cytotoxicity

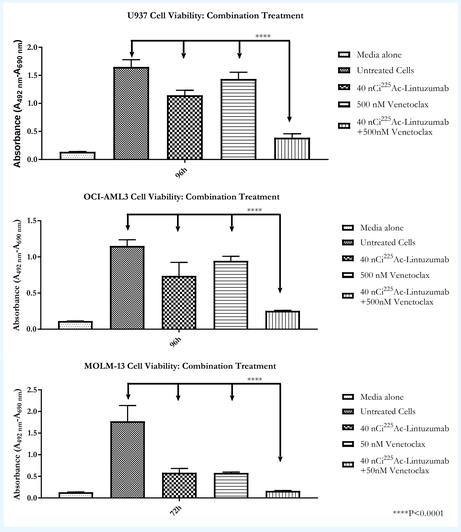


Figure 4: AML cell lines were treated with ^{225}Ac-Lintuzumab for 1 hour, washed, and then incubated with 50 nM (MOLM-13) or 500nM (U937 and OCI-AML3) venetoclax for the indicated time period. Cell viability was measured by XTT assay. In all three cell lines, combination treatment with ^{225}Ac-Lintuzumab and venetoclax resulted in increased cell death compared to individual treatment.

Figure 5: Impact of ^{225}Ac-Lintuzumab on Bcl-2 Family Protein Levels

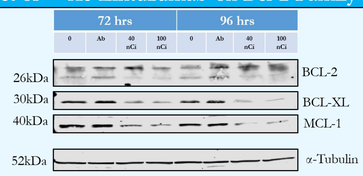


Figure 5: OCI-AML3 cells were treated with media alone or unlabeled Lintuzumab (Ab) or ^{225}Ac-Lintuzumab (40 and 100 nCi) for 1-hour and then washed three times and further incubated for 72hr and 96hrs. Cells were collected and lysed at indicated timepoint and western blotting was performed. Antibodies against Bcl-2, Bcl-XL, Mcl-1 were purchased from Cell Signaling. Western blot demonstrates that treatment of OCI-AML3 cells results in a modest impact on Bcl-2, Bcl-XL, and Mcl-1 protein levels. Similar results were also obtained by an ELISA assay.

Figure 6: Treatment with ^{225}Ac-Lintuzumab Results in Double-Stranded DNA Breaks

Percent Positive Cells Above Background	Untreated	Lintuzumab	40 nCi ^{225}Ac-Lintuzumab	100 nCi ^{225}Ac-Lintuzumab
U937 72 hrs	0.13	0.62	5.29	10.50
U937 96 hrs	0.80	1.30	15.9	21.2
OCI-AML3 72 hrs	0.15	0.13	13.2	16.7
OCI-AML3 96 hrs	0.18	0.61	23.2	25.4

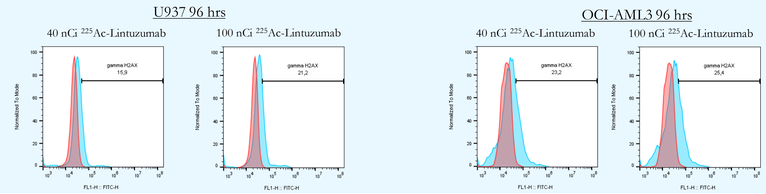
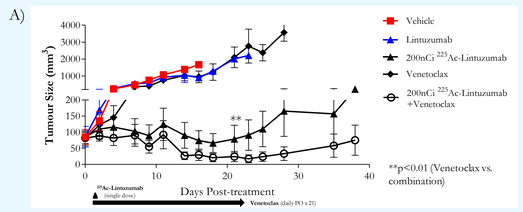


Figure 5: U937 and OCI-AML3 cells were treated with media alone or unlabeled Lintuzumab or ^{225}Ac-Lintuzumab (40 and 100 nCi) for 1 hour, washed three times, and further incubated for 72 and 96 hours. The presence of phosphorylated H2AX was measured through FACS (Upstate Cell Signaling) as an assay for the presence of double-stranded DNA breaks.

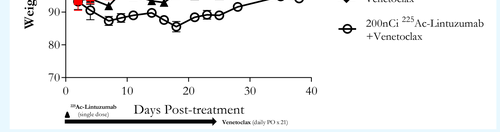
Figure 7: Tumor Regression and Survival Benefit of Venetoclax and ^{225}Ac-Lintuzumab Treatment Combination in Venetoclax Resistant OCI-AML3 Xenograft



**p<0.01 (Venetoclax vs. combination)

Treatment	D21 Response	D38 Response
Vehicle	5/5 Dead	5/5 Dead
Lintuzumab	4/5 Dead 1/5 PD	5/5 Dead -
200nCi 225Ac-Lintuzumab	- 1/5 PD 1/5 SD 2/5 PR 1/5 CR	1/5 Dead 2/5 PD - - 2/5 CR
Venetoclax	1/5 Dead 4/5 PD	5/5 Dead -
200nG 225Ac-Lintuzumab + Venetoclax	- 1/5 SD 2/5 PR 2/5 CR	2/5 PD - - 3/5 CR

Change in tumor volume vs D0: Progressive disease (PD) >30% increase; Stable disease (SD) +/- 30% change; Partial response (PR) >30% reduction; Complete response (CR) > 99% reduction.



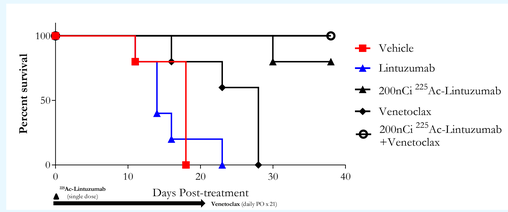


Figure 7: Female SCID mice were injected subcutaneously with OCI-AML3 cells and tumors were monitored until they reached approximately 100mm^3. Mice were then treated with a combination of single dose of ^{225}Ac-Lintuzumab and daily administrations of venetoclax (100mg/kg oral gavage) or individual treatment with ^{225}Ac-Lintuzumab or venetoclax. Mice were observed for (A) tumor progression, (B) weight, and (C) survival for 40 days. Combination treatment with ^{225}Ac-Lintuzumab and Venetoclax greatly increased tumor control and overall survival compared to individual treatment.

Summary

- Combination treatment of venetoclax resistant cell lines U937 and OCI-AML3 with venetoclax and ^{225}Ac-Lintuzumab greatly increases cell death in vitro compared to individual treatment.
- Mcl-1 protein levels are downregulated following ^{225}Ac-Lintuzumab treatment as measured by Western blot.
- ^{225}Ac-Lintuzumab treatment of cells results in a dramatic increase in double-stranded DNA breaks as measured by presence of phosphorylated H2AX.
- In a xenograft model using OCI-AML3 tumors in SCID mice, a single dose of ^{225}Ac-Lintuzumab followed by daily treatment with venetoclax resulted in robust tumor regression and improved overall survival compared to individual treatment.
- These results support a dual mechanism of action in which ^{225}Ac-Lintuzumab treatment induced DNA damage and subsequent Mcl-1 downregulation renders venetoclax resistant cell lines U937 and OCI-AML3 sensitive to venetoclax.
- These results highlight the potential benefit of combination therapy with radioimmunotherapy and venetoclax and support the advancement of this therapeutic strategy into clinical testing.