Application of High-Dimensional Spectral Cytometry in Conjunction with Ryvett Software to Evaluate *Ex-Vivo* Drug Responses in Acute Myeloid Leukemia (AML) Patient Samples

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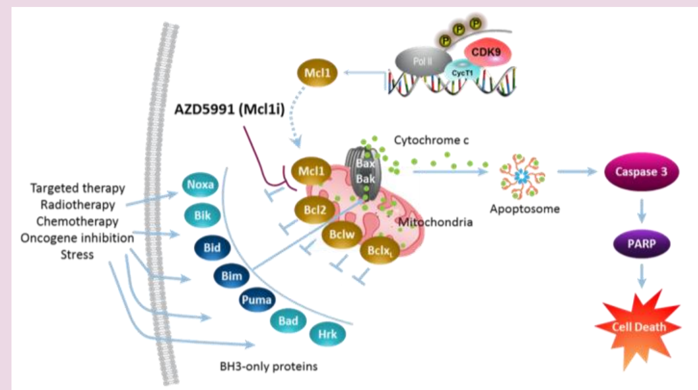
Background

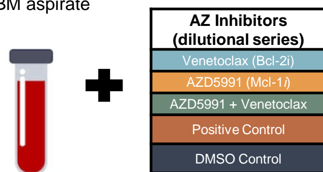
- Anti-apoptotic proteins are known to play a key role in the survival of various cancers. Numerous inhibitors of anti-apoptotic proteins such as Myeloid Cell Leukemia 1 (Mcl-1) and B-cell Lymphoma 2 (Bcl-2) and their combinations are currently being investigated in AML and other hematological malignancies.
- Mcl-1 has been shown to be upregulated after venetoclax treatment. It is a potential contributor to drug resistance mechanisms, as high expression of Mcl-1 prevents caspase activation in apoptosis. AZD5991 (Mcl-1*i*) is a BH3 mimetic inhibitor directly targeting the Mcl-1 protein that has shown potent antitumor activities in AML models.
- Acerta Pharma has developed an *ex-vivo* platform that measures inhibitor effects directly in whole blood (or bone marrow aspirate), providing more physiologically relevant information to support clinical studies. Due to the heterogeneity of AML, a high-dimensional 21-color, spectral flow cytometry assay was developed (using a Cytek Northern Lights) to explore deeper tumor biology and elucidate the effects of these inhibitors. The results shown in this study represent a small subset of samples that were used to explore and qualify this novel technology infrastructure.
- The complex dataset generated by this platform created challenges in data management and analysis. In order to address these challenges, Ryvett, an end-to-end cloud-based cytometry software with a centralized data repository, was used to design experiments, create acquisition-ready input files, and gate, quantify, and analyze single cell data.



Assay Methods

AZD5991 is an Mcl-1 inhibitor being evaluated in a Phase 1, multicenter study (NCT03218683) for multiple indications including AML. For AML patients, Peripheral Blood (PB) or Bone Marrow (BM) samples were collected just prior to treatment. Eleven predose samples (eight PB and three BM) from eight subjects were exposed to multiple *ex-vivo* treatments to study inhibitor effects using this platform.

Peripheral blood or BM aspirate



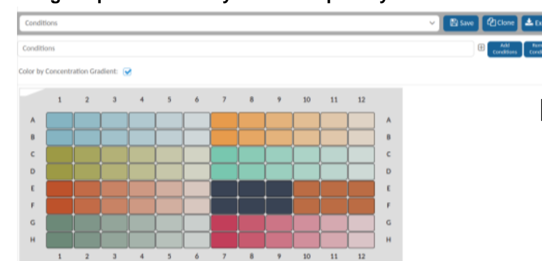
Incubate PB or BM + inhibitors for 6 hours

Fix whole blood / lyse red blood cells

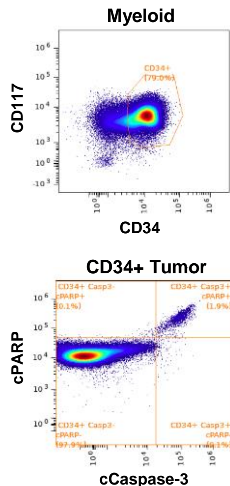
Permeabilize with methanol

Stain and acquire on flow cytometer

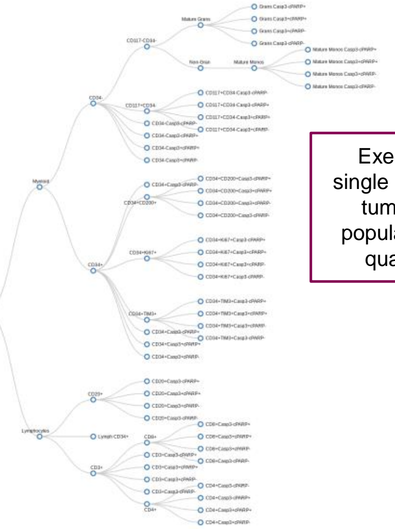
Design experiments in Ryvett and export Cytek instrument-ready file



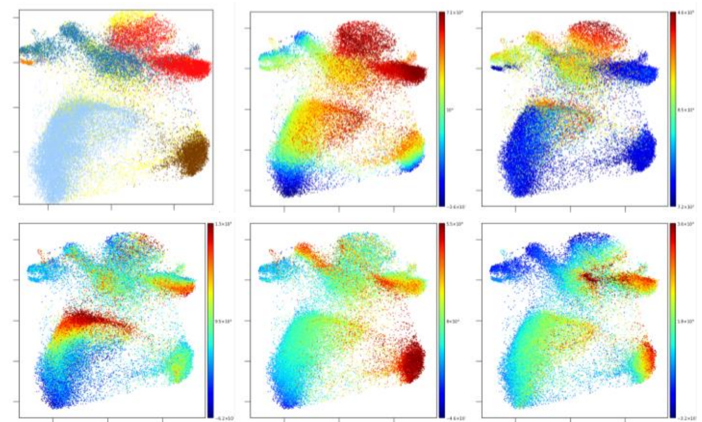
Analysis Methods



Gate for myeloid and lymphoid subsets



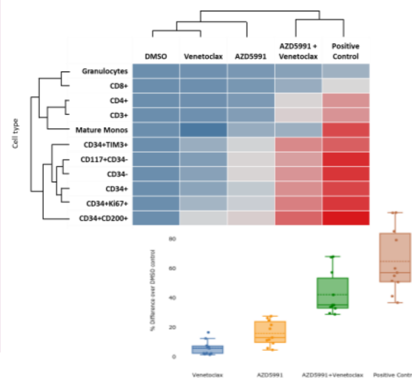
Execute and visualize single cell analysis: identify tumor and non-tumor populations to inform and quantify cell subsets

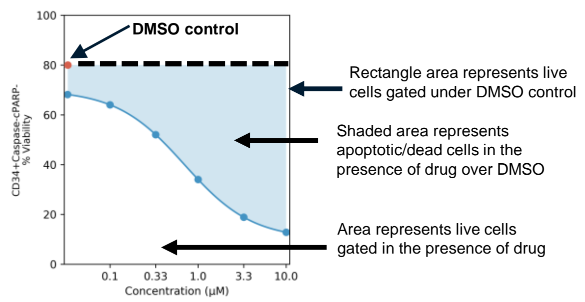


Quantify sensitivity of each sample to an inhibitor; centrally stored and auditable

- Expression of apoptotic proteins (Caspase-3 and cleaved PARP) and markers of proliferation (Ki67) and leukemic stem cells (TIM-3, CD200, CD123) were analyzed in a wide range of cellular subsets including leukemic blasts (CD34+, CD33+ or CD117+), mature monocytes (CD14+ or CD11b+), granulocytes (CD66b+), basophils (CD123+, HLA-DR-) and T cells (CD3+, CD4+ or CD8+).
- The data management and analysis workflow was executed in Ryvett:
  - Myeloid and lymphoid cell subsets were identified using gating functionality and further refined following single cell visualization.
  - The Uniform Manifold Approximation and Projection dimensionality reduction algorithm was applied to the myeloid cells to further visualize cell subsets and identify marker expression.
  - Sensitivity of a sample to an inhibitor (or a combination) was quantified relative to dimethyl sulfoxide (DMSO) using a method that accounted for background apoptosis and integrated all the drug concentrations.
  - This quantified data was stored within Ryvett allowing it to be auditable and traceable along with the associated single cell data.

Analyze quantified data



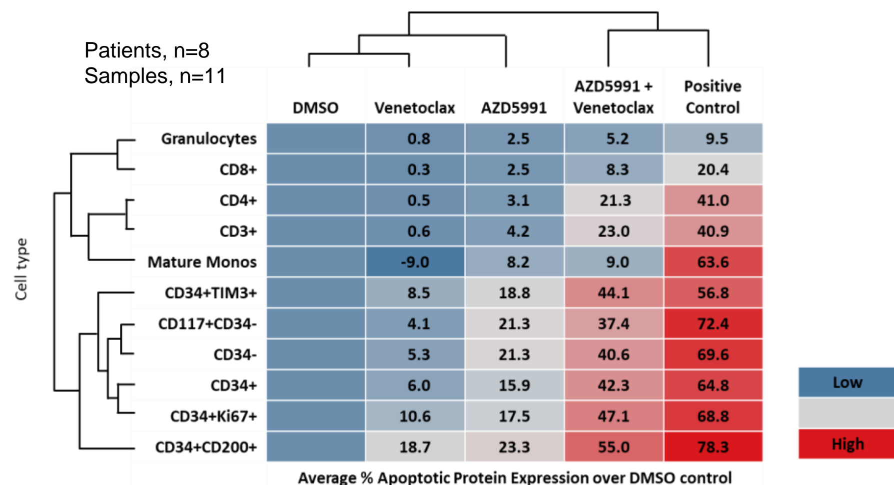


$$\%\ \text{apoptosis over DMSO control} = 100 * \frac{(AUC_{DMSO} - AUC_{drug})}{AUC_{DMSO}}$$

AUC is computed using trapezoid rule. For data with replicate points for each conc, the values are averaged first before computing AUC.

Results

Figure 1. Aggregate analysis of cleaved Caspase-3 and cleaved PARP expression in AML patient samples reveals sensitivity of CD34+ tumor subsets to AZD5991 +/- venetoclax compared to other cell populations

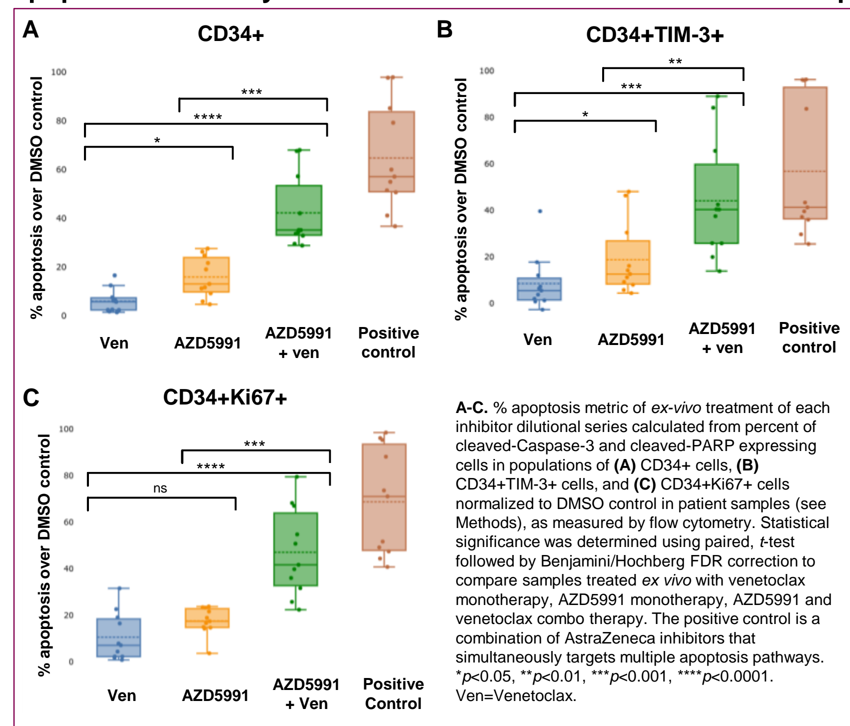


Cell type	DMSO	Venetoclax	AZD5991	AZD5991 + Venetoclax	Positive Control
Granulocytes		0.8	2.5	5.2	9.5
CD8+		0.3	2.5	8.3	20.4
CD4+		0.5	3.1	21.3	41.0
CD3+		0.6	4.2	23.0	40.9
Mature Monos		-9.0	8.2	9.0	63.6
CD34+TIM3+		8.5	18.8	44.1	56.8
CD117+CD34-		4.1	21.3	37.4	72.4
CD34-		5.3	21.3	40.6	69.6
CD34+		6.0	15.9	42.3	64.8
CD34+Ki67+		10.6	17.5	47.1	68.8
CD34+CD200+		18.7	23.3	55.0	78.3

% apoptosis metric of *ex-vivo* treatment of each inhibitor dilutional series calculated from percent of cleaved-Caspase-3 and cleaved-PARP expressing cells in all populations normalized to DMSO control in patient samples (see Methods). Average value shown of all samples in data set (n=11). Y-axis dendrograms are self-rearranging to illustrate similarity between cell types of sensitivity to inhibitors. The positive control is a combination of AstraZeneca inhibitors that simultaneously targets multiple apoptotic pathways.

- This platform allows for efficient interrogation of multiple CD34+ tumor cell subsets such as CD200+, CD117+, TIM-3+ and Ki67+ and also further delineates lymphocytes into CD4+, CD8+, and CD20+ (not shown due to low cell count consistent with AML), as well as granulocytes and monocytes.
- Based on a limited number of patients (n=8), tumor cells are more sensitive to AZD5991 monotherapy and the combination with venetoclax than lymphocytes, mature monocytes and granulocytes.
- Increased expression of cleaved Caspase-3 and cleaved PARP illustrate synergy of AZD5991 combination with Venetoclax when compared to monotherapy conditions

Figure 2. Analysis of CD34+ tumor cell subsets shows increased apoptosis sensitivity to AZD5991 + venetoclax than to monotherapy



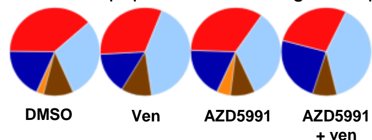
A-C. % apoptosis metric of *ex-vivo* treatment of each inhibitor dilutional series calculated from percent of cleaved-Caspase-3 and cleaved-PARP expressing cells in populations of **(A)** CD34+ cells, **(B)** CD34+TIM-3+ cells, and **(C)** CD34+Ki67+ cells normalized to DMSO control in patient samples (see Methods), as measured by flow cytometry. Statistical significance was determined using paired, *t*-test followed by Benjamini/Hochberg FDR correction to compare samples treated *ex vivo* with venetoclax monotherapy, AZD5991 monotherapy, AZD5991 and venetoclax combo therapy. The positive control is a combination of AstraZeneca inhibitors that simultaneously targets multiple apoptosis pathways. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Ven=Venetoclax.

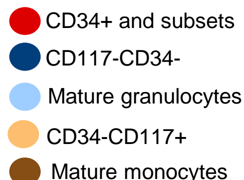
- Consistent with prior Venetoclax treatment in most of these patients, tumor cells were observed to be more sensitive to AZD5991 than Venetoclax. Specifically, AZD5991 promotes more apoptotic protein expression in the overarching CD34+ cells and CD34+TIM-3+ subpopulation.
- Synergistic effects of AZD5991 + venetoclax treatment are seen in CD34+ cells and downstream subpopulations including more stem-cell like (CD200+, TIM-3+) and proliferating (Ki67+) subsets that can play a critical role in maintaining disease.

Results

Figure 3. Minimal cell reduction observed at 6 hours, highlighting the utility of apoptotic biomarkers cleaved-Caspase-3 and cleaved-PARP

Ratio of cell populations in a single sample





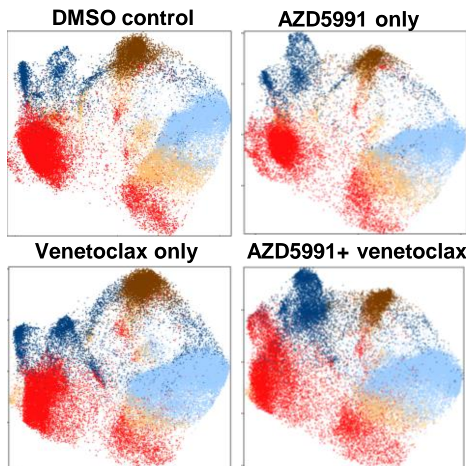


Figure 4. Expression of apoptotic cPARP marker

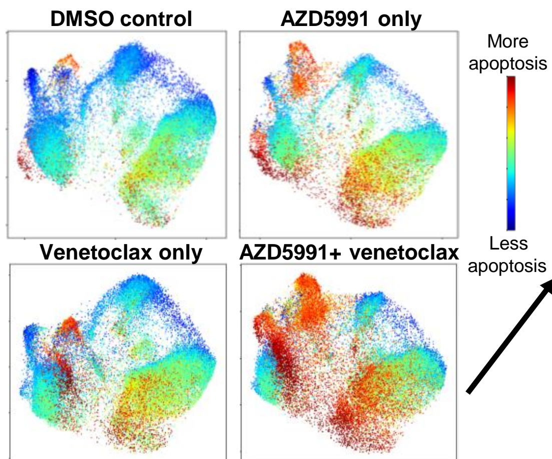
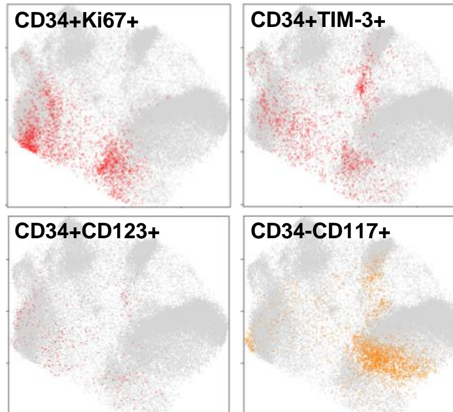


Figure 5. Leukemic blasts (CD200+, TIM-3, CD123+) and proliferating (Ki67+) cells are apoptotic with AZD5991+ venetoclax combination



- AZD5991 monotherapy and combination with venetoclax modestly reduce cell counts in blast tumor populations (Figure 3). At the 6-hour timepoint and at the concentration used, many cells are still expected to be mid-apoptosis.
- Apoptosis marker (cPARP) expression in the 4 different treatment conditions shows partial apoptosis in the tumor populations treated with venetoclax or AZD5991 monotherapy but significantly higher apoptosis with combination treatment (Figure 4).
- Combination treatment across cell subsets of leukemic blast cells (Figure 5) causes apoptosis in a majority of proliferating (Ki67+) and stem cell blast subsets (TIM-3+ and CD123+)

Conclusions

- We have developed a novel drug sensitivity assay in conjunction with spectral flow and single cell analysis which has allowed for deeper exploration of AML immune cell types, and provided novel insights into tumor biology. It also has the potential to profile and prioritize drug treatments.
- Based on a limited set of clinical samples from AML patients, strong synergy was seen with the AZD5991 plus venetoclax combination
  - The CD34+ population and its downstream subsets exhibited higher rates of apoptosis as indicated by cleaved-Caspase-3 and cleaved-PARP.
  - Consistent with what has been reported for AML cell lines[1]
- Further enhancement of the antibody panel to include additional markers of myeloblast differentiation (e.g. basophil and additional tumor markers) is underway.

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References

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Conflicts of Interest
MH and **AW** are employed by Acerta Pharma and have received equity ownership from Acerta Pharma and/or AstraZeneca. **TY** is employed by AstraZeneca and has received equity ownership from AstraZeneca. **NW** and **SP** are employed by Qognit Inc., the creator of Ryvett software, and are consultants to Acerta Pharma/AstraZeneca. **MG** was formerly employed by Acerta Pharma and has received equity ownership from Acerta Pharma and/or AstraZeneca

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