Differentiation of Azacitidine vs Oral AZA: Oral AZA results in a sustained hypomethylation and targets leukemic stem cells

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Acute Myeloid Leukemia (AML) is a hematological disorder characterized by the uncontrolled proliferation of incompletely differentiated myeloid stem cells. Despite recent advances in therapy, high rates of clinical relapse, even in patients who achieve complete remission, remains a problem. One of the strategies to prolong remission in AML is to employ effective maintenance therapies. Oral Azacitidine (Oral-AZA; CC-486) is the first and only currently approved maintenance therapy in AML. However, the mechanism of action by which Oral-AZA is differentiated from Injectable-AZA, used in AML induction therapy, remains unclear. In this work, we attempted to differentiate Injectable vs Oral-AZA.

***In vitro* modelling of Oral-AZA vs Injectable-AZA:** To model an Injectable-AZA-like regimen in vitro, we used the clinically relevant Injectable-AZA concentration (1 μM) as a single dose (HELD – High Exposure Limited Duration). A fractionated dose of 0.2 μM each day over 5 days (LEED – Low Exposure Extended Duration) was used to model Oral-AZA.

Injectable-AZA-like dosing leads to a rapid activation of the integrated stress response (ISR) pathway likely through RNA incorporation of azacitidine: HELD but not LEED demonstrated acute anti-proliferative effects in sensitive AML cell lines suggesting a non-hypomethylation mediated/stress response driven effect. This effect was rescued by ISRIB, an ISR inhibitor. Consistent with this, we observed robust ATF4 activation as early as 6 hours that was sustained up to 24 hours in HELD. LEED on the other hand induced modest and transient ATF4 activation. Thus, an Injectable-AZA-like regimen activates the ISR pathway robustly than an Oral-AZA-like regimen. Interestingly, decitabine, a DNA incorporating cytidine analog did not activate ISR suggesting that azacitidine, the RNA incorporating cytidine analog, drives ISR through its ability to incorporate into RNA.

Oral-AZA-like dosing leads to a sustained loss of DNMT1 resulting in a more durable hypomethylation: DNMT1, the target of Azacitidine, was rapidly depleted (about 90% depletion) within 24 hours in both HELD and LEED dosing. However, LEED produced a more sustained DNMT1 loss, up to 7 days. On the other hand, in HELD, DNMT1 protein levels recovered 96 hours post-dosing. Given this difference in the levels of DNMT1, we hypothesized that LEED would lead to a more durable hypomethylation. To further validate this, we performed whole genome bisulfite sequencing (WGBS) in 3 AML cell lines (OCI-AML2, MV-4-11 and SKM1) at 48- and 96-hours post-start of HELD and LEED dosing. Consistent with the DNMT1 depletion kinetics, at 48 hours we observed almost 75% hypomethylation in both HELD and LEED. At 96 hours, HELD demonstrated a bounce back effect and reverted to 50% hypomethylation. In contrast, LEED showed up to 85% hypomethylated sites, demonstrating the durability of the hypomethylation mediated by an Oral-AZA-like (LEED) regimen.

Oral-AZA-like dosing differentiates leukemic stem cells (LSCs) towards a more mature phenotype: Elimination or differentiating LSCs has been postulated to be an effective strategy in AML maintenance therapy. Using an *in vitro* LSC model (OCI-AML-20) coupled with flow cytometry, we identified that LEED results in a greater depletion (2-fold more) of LSCs (CD34+/38- or 38 low) and enrichment towards a more differentiated phenotype (CD34+/38+) than HELD. To further validate these observations, we performed single cell RNAseq with the LSC model at different timepoints (3, 5 and 7 days). Data were analyzed using the widely adopted Van Galen classifier that identifies different leukemic myeloid cell lineages. Compared to control cells, at day 7, treatment resulted in an increase of GMP and Promonocytes with those differences more pronounced under Oral-AZA-like (50% GMP and 20% promonocytic) than the Injectable-AZA-like regimen (28% GMP and 12.5% promonocytic). Thus, our data reveal an LSC depletion mechanism associated with Oral-AZA.

Conclusion: Our work demonstrates that an Injectable-AZA-like regimen mediates cytotoxicity through an early stress-response driven effect, consistent with acute growth inhibition associated with Ven/Aza combinations in preclinical setting. Oral-AZA on the other hand leads to a more sustained effect that results in a differentiation inducing effect on the leukemic stem cell population likely through durable hypomethylation.

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