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Extended exposure to low doses of azacitidine induces differentiation of leukemic stem cells through activation of myeloperoxidase

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Abstract
Oral azacitidine (Oral-Aza; CC-486) treatment results in longer median overall survival (OS) (24.7 vs 14.8 months in placebo) in patients with acute myeloid leukemia (AML) in remission after intensive chemotherapy. The dosing schedule of Oral-Aza (14 days/28-day cycle) allows for low exposure of azacitidine for an extended duration thereby facilitating a sustained therapeutic effect. However, the underlying mechanisms supporting the clinical impact of Oral-Aza in maintenance therapy remain to be fully understood. In this preclinical work, we explore the mechanistic basis of Oral-Aza/extended exposure to azacitidine through in vitro and in vivo modeling. In cell lines, extended exposure to azacitidine results in sustained DNMT1 loss, leading to durable hypomethylation, and gene expression changes. In mouse models, extended exposure to azacitidine, preferentially targets immature leukemic cells. In leukemic stem cell (LSC) models, the extended dose of azacitidine induces differentiation and depletes CD34+CD38- LSCs. Mechanistically, LSC differentiation is driven in part by increased myeloperoxidase (MPO) expression. Inhibition of MPO activity either by using an MPO specific inhibitor or blocking oxidative stress, a known mechanism of MPO, partly reverses the differentiation of LSCs. Overall, our pre-clinical work reveals novel mechanistic insights into oral-Aza and its ability to target leukemic stem cells.

INTRODUCTION
AML is a disease of the elderly with a median age at diagnosis >68 years. Unfavorable cytogenetics and associated comorbidities are among the factors that make elderly patients unfit/ineligible for intensive chemotherapy, known as the 7+3 regimen (1-3). As a result, targeted and less-intensive treatments are used as first line therapy for many of these patients. Venetoclax/Azacitidine (Ven/Aza) is one such treatment regimen that has improved outcomes in chemotherapy ineligible newly diagnosed AML patients (4, 5). The backbone of this regimen, Aza, is a hypomethylating agent that incorporates into RNA and DNA. DNA-incorporated Aza leads to loss of DNMT1 and therefore, genome-wide hypomethylation (6). Through hypomethylation dependent/independent effects, Aza impacts multiple cellular processes such as apoptosis, DNA damage response, tumor suppressor re-expression, immune modulation, and more (7). Given the genomic and epigenomic dysregulation and heterogeneity in AML, the broad hypomethylating effect and multitude of mechanisms impacted by Aza could be one of the keys to its clinical efficacy. Venetoclax selectively inhibits the anti-apoptotic protein, BCL-2 (8). On the other hand, many AML patients eligible for intensive chemotherapy achieve
complete remission with the 7+3 regimen (3). However, most of these patients on chemotherapy as well as Ven/Aza therapy eventually relapse (9-11).

A multicenter study identified longer relapse free survival following first remission as one of the key parameters for better prognosis (12). Thus, better maintenance therapy is critical for AML patients. The QUAZAR AML-001 study demonstrated that maintenance therapy with Oral-Aza significantly prolonged overall and relapse-free survival (13,14). Interestingly, single agent injectable-Aza did not provide a significant overall survival benefit in maintenance therapy in a similar patient population (15, 16). The key translational question posed by this observation is, what specific mechanisms are associated with the oral formulation that may contribute to the observed clinical benefit in AML maintenance. To further explore this, we modelled injectable- and Oral-Aza in our preclinical studies and explored the mechanism of action of Oral-Aza. Equally important is to identify similarities between the two dosing regimens for potential future investigations of Oral-Aza with agents that could combine well with injectable-Aza.

METHODS

Human and Mouse Cell Lines
AML cell lines were cultured in RPMI1640 (SKM1), Iscove’s Modified Dulbecco’s Medium (IMDM; OCI-AML2, OCI-AML20, MV411) or α-MEM (OP9, OCI-AML3, OCI-AML5) supplemented with 10% or 20% fetal bovine serum (FBS), sodium pyruvate, glutamine, and penicillin-streptomycin. OP9 cells were further supplemented with beta-mercaptoethanol (β-ME) and OCI-AML20 with β-ME and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Flow Cytometry
For OCI-AML20, CD33, and CD45, stains were used to distinguish OP9 cells (mouse) and OCI-AML20 cells (human). OCI-AML20 were then assessed for differentiation and changes in leukemic stem cell (LSC) population with the following markers: Live/Dead stain, CD34 and CD38. Flow cytometry was performed in a BD LSRFortessa™ X-20.

MPO inhibition
OCI-AML20 cells were treated with azacitidine (conventional or extended dose) alone or in combination with 10 µM MPO-IN-28 (dosed on days 1 and 4). Flow analysis was done on day 7.
Cytokine Array
OCI-AML20 cells were seeded at 0.5 $\times$ 10^6 cells/mL and treated with azacitidine, conventional or extended dosing. Supernatant from the cells were collected on days 3, 5, and 7. Cytokine array was performed using Proteome Profiler Human XL Cytokine Array Kit (biotechne, ARY022B), according to manufacturer’s recommendation except that membranes were incubated with IRDye 800CW Streptavidin (Licor, 926-32230) to allow fluorescent detection of the cytokines. Membranes were imaged with Odyssey CLx (Licor).

Primary AML cell culture and CD34+ cells enrichment
Frozen primary patient sample (mutations in BCOR BCORL1 CDKN2A IKZF1 NOTCH1 RUNX1 SF3B1 STAG2 TET2 TP53) were quickly thawed and followed by CD34+ cells isolation (Stemcell Technologies, 17856) according to the manufacturer’s protocol. Flow cytometry was used to verify enrichment of CD34+ population before treatment initiation. Cells were resuspended to a concentration of 0.5 $\times$ 10^6 cells/mL in X-VIVO 10 medium (Lonza, 04-380Q) supplemented with 20% BIT 9500 serum substitute (STEMCELL Technologies, 09500) and cytokines: IL-6 (10 ng/ml; PeproTech, 200-06), IL-3 (10 ng/ml; PeproTech, 200-03), stem cell factor (50 ng/ml; Peprotech, 300-07), FLT3-Ligand (50 ng/ml; PeproTech, 300-19), granulocyte colony-stimulating factor (10 ng/ml; PeproTech, 300-23), and TPO (25 ng/ml; PeproTech, 300-18). The cells were dosed with Aza - extended or conventional and flow analysis was performed on day 7 day with live/dead stain, CD34, and CD38 antibodies.

In vivo Studies
For in vivo intrafemoral engraftment studies, we modeled injectable-Aza and oral-Aza doses with 3 mg/kg qd x 5 days and 1 mg/kg qd x 15 days, respectively (intraperitonially in both cases, modified from Vu et al. (13). Twelve-week female immunodeficient NOD.CB17-Prkdc<sup>scid</sup>/J (NOD-SCID)(17) mice used for the transplantation of primary AML cells were obtained from the Ontario Cancer Institute (Toronto, ON, Canada). All animal studies were performed in accordance with the Ontario Cancer Institute Animal Use Protocol (AUP): # 1251.33 (NOD-SCID).

For the syngenic mouse model: C1498 (ATCC® TIB-49™) murine acute leukemia cells (American Tissue Culture Collection) expressing firefly luciferase and GFP (designated C1498-Luc3-GFP) were developed at Charles River Laboratories. Mice were injected with 1 $\times$ 10^6 C1498-Luc3-GFP tumor cells via the tail vein. Animals were randomized into treatment groups
based on body weight on Day 1 post-injection. All drug treatments were initiated on Day 5 and were administered once daily (QD). Vehicle (PBS) and azacitidine were administered intraperitoneally.

For the chimeric AML model: FLT3-ITD, TET2, and Lys-M cre mice were bred to generate a heterologous genotype of FLT3-ITD-/+TET2-/+LysM-cre-/+.. Bone marrow chimeras were generated by irradiating mice in an x-ray irradiator at a lethal dose of 9 Gy. Bone marrow from FLT3-ITD-/+TET2-/+LysM-cre-/+ (CD45.2) was adoptively transferred via intravenous injection into 8-week-old CD45.1 congenic disparate recipient mice. Mice were monitored for chimerism after week 8 post-adoptive transfer. Azacitidine was administered intraperitoneally.

RESULTS

Extended-Dose Aza Leads to Durable Hypomethylation and Sustained Gene Expression independent of the Integrated Stress Response Pathway

While both injectable- and Oral-Aza have the same active ingredient, their clinical dose and schedules are different, and they do not have similar bioavailability, pharmacokinetic (PK) or pharmacodynamic (PD) profiles (18, 19). In a phase I study, compared with non-responders, patients who achieved clinical response with Oral-Aza had greater hypomethylation as measured by global demethylation scores (19). This indicated that the dosing schedule might impact the hypomethylative effect of Aza. To test this hypothesis, we modeled injectable and oral-Aza based on a recent study by Vu and colleagues (20). They modeled the clinical exposures of injectable- and Oral-Aza using a “high exposure, limited duration” (HE-LD) regimen mimicking injectable-Aza and a “low exposure, extended duration” (LE-ED) mimicking Oral-Aza hereafter referred to as conventional and extended dose respectively.

To understand the mechanistic basis of greater hypomethylation by oral-Aza, we treated three different AML cell lines with the *in vitro* modeling of the conventional versus extended dosing regimens. In these Aza treated samples, genome wide methylation was measured by whole genome bisulfite sequencing and gene expression changes by RNA-seq (Fig. 1A) on days 3 and 5. While day 3, showed greater genome-wide hypomethylation in the conventional dose in 2 of the 3 cell lines (Fig. 1 B, C, and D [upper panels]), by the end of treatment (day 5), the extended-dose Aza regimen demonstrated durable hypomethylation in all three cell lines relative to the conventional Aza (p<0.001) (Fig. 1B, 1C, and 1D [lower panels] (based on beta score greater than 0.7) and supplementary Fig. S1A, supplementary table 1). This suggested
progressive hypomethylation in the extended dose but not in the conventional dose. Similarly, gene expression changes were greater/progressive in the extended dose at the end of treatment (day 5) (Fig. 1E). Intrigued by the sustained effects of the low-dose Aza, we explored the status of DNMT1, which is degraded through entrapment by Aza thereby facilitating hypomethylation (6). Biochemical analysis revealed loss of DNMT1 (90% reduction) at early timepoints (Fig. 1F). On day 7, DNMT1 levels recovered to nearly 90% with the conventional dose (Fig. 1F) but not in the extended dose. Thus, the extended dose, albeit at lower exposures of Aza, facilitates prolonged loss of DNMT1 and thereby durable hypomethylation. Of note, while up to day 10, DNMT1 levels continue to be low in extended Aza treatment, there is about 20-50% cell death at these timepoints raising the possibility that at timepoints beyond day 7, cell death could contribute to loss of DNMT1 (data not shown).

To further understand the sustained hypomethylation of the extended-dose Aza, we analyzed the pharmacodynamic (PD) basis in an in vivo study measuring [14C] azacitidine incorporation in PBMCs after intraperitoneal administration to male C57BL/6 mice. These mice were treated with the two dosing regimens to mimic the conventional dose (3mg/kg, QDx5) and extended dose (1mg/kg, QDx15) regimens of Aza (supplementary Fig. S1B) with both regimens receiving the same cumulative dose of Aza. Results showed that the relative incorporation of azacitidine into DNA was greater in the extended dose based on relative AUCs (Fig. 1G). Thus, the continued exposure of Aza resulted in a sustained therapeutic effect through greater nucleic acid incorporation thereby explaining the prolonged loss of DNMT1.

RNA incorporation of Aza is a potential mechanism through which, integrated stress response (ISR), one of the known mechanisms of action of Aza (21), is activated. In a recent study, this has been postulated as a mechanistic basis for synergy with venetoclax (22). To investigate if the extended-dose also activates ISR, we treated MV-4-11, SKM-1, and AML-193 cell lines with 0.2 µM or 1 µM Aza in short-term (24 hours) experiments (Fig. 1H). As expected, 1 µM Aza, mimicking conventional-Aza, resulted in increased levels of eIF2α phosphorylation as well as ATF4, the core and effector of ISR, respectively (23). With the 0.2 µM dose, we observed slightly lower levels of phospho-eIF2α and ATF4. In addition, treatment with ISRIB (24), a known inhibitor of ISR, reversed ATF4 expression without impacting DNMT1 loss. To investigate if ISR could be activated at later stages of the extended dose, we performed a time-course experiment over a period of 7 days (Fig. 1I). While the extended dose was able to induce ATF4 expression, it did not result in activation of C/EBP Homologous Protein (CHOP), a downstream effector of ATF4 that leads to cell death (25) which was observed in the 1 µM dose
at 24 hours. These results indicate that while both the conventional and extended dosing trigger a stress response, the extended dose is not robust enough to mediate cell death through the ISR pathway.

Taken together, the above observations indicate that while conventional Aza works through activation of stress response and hypomethylation mediated changes, the extended dosing of Oral-Aza results in sustained gene expression driven temporal effects that could be a key differentiating factor between injectable- and Oral-Aza.

**Extended dosing of Aza preferentially targets bone marrow blasts than conventional Aza**

To further investigate the therapeutic impact of the above observed differences, we investigated the effect of the two dosing regimens in an in vivo syngeneic and PDX model of AML. C1498-Luc3-GFP cells were injected into the tail vein of syngeneic mice which were then treated with the two dosing regimens of Aza. Ventral bioluminescence imaging (BLI) of luciferase-expressing tumor cells demonstrated that both regimens of azacitidine significantly decreased tumor burden at 15,18, and 21-days post-injection (supplementary Fig. S2A). At day 25 post-injection, flow cytometry analysis demonstrated that both dosing regimens significantly decreased the percentage of GFP+ C1498 tumor cells in peripheral blood (supplementary Fig. S2B). Both Aza regimens similarly increased survival of tumor-bearing mice, compared to vehicle controls (Fig. 2A) suggesting that both regimens are equally effective in reducing disease burden.

To further validate these in vivo observations, we used an IDH1R132H patient-derived xenograft (PDX) AML model, CTG-2227 (26). Cells were injected into the tail vein of sublethally irradiated, immunocompromised mice (NOG-EXL) and the animals were treated with the two dosing regimens. Tumor burden was assessed by measuring spleen weight at the end of the study. Similar to the syngenic model, both dosing regimens caused significant and comparable decreases in spleen weight compared to vehicle treated animals (Fig. 2B). Taken together, the syngeneic and PDX models indicate that despite the difference in dosing schedule, both the Aza dosing regimens are equally effective against bulk AML cells and contribute to lowering disease burden.

Given that Oral-Aza is effective in maintenance therapy where leukemic stem/progenitor cells are one of the drivers of relapse, we tested the two dosing regimens in a chimeric AML model. Bone marrow from FLT3-ITD; TET2; LysM-cre compound heterozygous mice on a CD 45.2 background was adoptively transferred to CD45.1 wild type (WT) recipient mice. Again, the extended Aza dosing was as effective as the conventional dosing at resolving splenomegaly
(Fig. 2C), a strong indicator of AML progression; However, the extended Aza dosing significantly decreased blasts in the bone marrow, whereas conventional dosing did not (Fig. 2D). These data suggest that the extended dosing regimen more effectively controls bone marrow disease in this model.

Extended-dose Aza Induces Differentiation/Elimination of LSCs

Leukemic stem cells are primitive hematopoietic cells related to the origin and relapse in AML (27, 28). To investigate the impact of the Oral-Aza/extended dosing on the LSC compartment, we utilized an in vitro LSC model, OCI-AML20 (29). Extended Aza dosing resulted in depletion of LSCs (CD34+/38- or 38 low) and concomitant enrichment of CD34+/38+ cells at day 7 (Fig. 3A (representative flow plot) and supplementary Fig. S3A (replicate data). While the conventional Aza dosing also resulted in depletion of CD34+38- cells, the extended Aza dosing had greater depletion of LSCs in response to the extended-dose Aza. Notably, no overt cell death/apoptosis observed in these cells (supplementary Fig. S3B). To further validate the differentiation phenotype, we treated a CD34+ cells enriched primary AML sample (TP53 and TET2 mutated) with both dosing regimens and identified differentiation of CD34+ cells to CD34- cells (Fig. 3B and supplementary Fig. S3C). To further substantiate these observations, we performed single cell RNA-seq in OCI-AML20 cells at different timepoints (3, 5, and 7 days). Data were analyzed using a previously described classifier to identify leukemic myeloid cell lineages (30). Compared to untreated cells, the extended dosing resulted in an increase of granulocyte-monocyte progenitor (GMP) and myeloid cells indicating induction of differentiation in immature cells. While the conventional dosing also resulted in similar change in cell type composition, the extended dosing regimen was associated with significantly greater differentiation compared with the conventional regimen (Fig. 3C).

To test the effects of Oral-Aza like dosing on leukemic progenitors in vivo, we leveraged a mouse xenograft model (NOD/SCID). Following injection of AML patient-derived peripheral blood mononuclear cells (PBMCs) into the right femur, the mice were treated with Aza regimens mimicking the injectable/conventional dose (3 mg/kg x 5 days) or the oral/extended dose (1 mg/kg x 15/21 days) (Fig. 4A). At day 42, animals were sacrificed, and primary engraftment was measured in the left femur/distal site. Both the conventional and extended dosing reduced engraftment by about 10-fold relative to the control treatment (Fig. 4B).

To further validate the differentiation phenotype observed with the extended dose, we analyzed the human PBMCs that remained at the site of injection (right femur) in the primary engraftment
experiment. Similar to the engraftment, the degree of differentiation induction on progenitors was similar in both regimens. Most of the cells in the sample tested were arrested in the GMP-like phase and both regimens of Aza induced the differentiation of >50% of these cells into a common myeloid progenitor (CMP) or megakaryocyte-erythrocyte progenitor (MEP) like phenotype (Fig. 4C-E). There was also reduction of CD33+ 123+ stem/progenitor cells at the site of injection (Fig. 4F). Notably, there was no overt loss of weight in the animals (Fig. 4G).

Taken together, our data based on an in vitro LSC model suggest that the efficacy of Oral-Aza in AML maintenance is in part linked to the ability of the extended dosing schedule to target LSCs. In addition, the in vivo model demonstrates that Oral-Aza induces differentiation of leukemic stem and progenitor cells.

**Extended-Dose Aza induces differentiation through increased myeloperoxidase activity**

To understand the molecular determinants of the greater degree of differentiation occurring in the LSC compartment in the extended dose, we sorted CD34+/38- cells from OCI-AML20 cells (Fig. 5A) and treated them with the two Aza dosing regimens and performed bulk RNA-seq at day 5. As expected, pathway analysis (GO process) revealed that the most significantly upregulated pathway was cellular differentiation in the CD34+38- cells upon 5 days of extended dosing but not in the conventional dosing (Fig. 5B).

To investigate drivers of the upregulated cell differentiation pathway, we analyzed secreted factors from cell culture supernatants of OCI-AML20 cells treated with both the Aza dosing regimens in a feeder layer free culture using the Proteome Profiler™ antibody array. Among the differentially secreted factors, myeloperoxidase (MPO), a hallmark enzyme of the myeloid lineage was the secreted factor that was also the most upregulated by gene expression analysis in LSCs that were treated with the extended dose Aza (supplementary Fig. S4A). In addition, Western blot analysis confirmed that MPO is upregulated in OCI-AML20 cells as well as in CD34+ cells from a primary sample (TP53 and TET2 mutated) in response to the extended dose (Fig. 5C). Given MPO’s association to myeloid differentiation, we hypothesized that upregulation of MPO could be implicated in extended Aza-induced LSC differentiation. To test this hypothesis, we used an inhibitor of MPO, MPO-IN-28 (31) and treated OCI-AML20 cells with MPO-IN-28 and the both the Aza doses. Inhibition of MPO countered the differentiation induction by extended dose Aza (Fig. 5D and supplementary Fig. S4B) by more than 20% reduction in the non-LSC population (CD34+38+ or CD34-). Being a peroxidase, MPO has been shown to induce reactive oxygen species (ROS) generation in myeloid cells (32), a potential
mechanism that has been shown to induce differentiation of LSCs (33, 34). To investigate if MPO induced ROS could facilitate differentiation in LSCs, OCI-AML20 cells were concurrently treated with N-acetylcysteine (NAC) and the two Aza dosing regimens. ROS scavenging with NAC, blocked differentiation of LSCs with the extended dose but not with the conventional dose further validating our hypothesis (Fig. 5E and supplementary Fig. S4C). Taken together, our results suggest that extended dose Aza induces differentiation of LSCs through upregulation of MPO and ROS.

DISCUSSION

In AML maintenance therapy, the primary goal is to eliminate residual leukemic cells in the bone marrow thereby eliminating minimal residual disease (MRD) and reducing the risk of relapse. However, rare therapy resistant cells that reside within the MRD, also known as leukemic stem cells, can drive clonal leukemic repopulation resulting in relapse (28, 35). Thus, understanding the biology of these LSCs and identifying therapeutic agents that effectively target LSCs during induction or maintenance therapy would be important in reducing rates of relapse in AML. While oral-Aza is effective in AML maintenance therapy, the mechanistic basis was not clear. Our pre-clinical work, for the first time demonstrates oral-Aza’s ability to target LSCs.

Even though both injectable and oral-Aza have the same active ingredient, the dosing schedule results in varying PK and PD profiles as well as therapeutic effects. Based on the pre-clinical data presented here, Oral-Aza acts primarily through sustained hypomethylation mediated gene expression changes and injectable-Aza acts through stress response as well as hypomethylation (not as sustained as oral-Aza). Consistent with our observations, a study with low-risk MDS patients reported sustained hypomethylation imparted by oral-Aza over the entire treatment cycle (up to 21 days). In addition, patients administered with oral-Aza and with resultant reduction in DNA methylation had better hematologic response (36). Similarly, sustained hypomethylation with oral-Aza were observed in another independent study (19).

Given the biochemical differences observed (Fig. 1) between the two dosing regimens, our in vivo studies yielded interesting similarities and differences (Fig. 2) highlighting the functional impact of the two dosing regimens based on differentiation state of the cell. Both oral and injectable Aza were equally efficacious in targeting bulk AML cells in the syngeneic and the PDX model, both of which used bulk AML cells. However, when we looked at less differentiated bone marrow blast cells in the FLT3-ITD; TET2 model, the extended dose was effective in targeting
this less differentiated population. When we applied this finding to an in vitro LSC model (OCI-AML20), we observed similar results and demonstrated that oral-Aza is more effective in eliminating immature myeloid cells and inducing differentiation of leukemic stem cells through upregulation of myeloperoxidase. Myeloperoxidase, being a ROS generator, in turn likely acts through reactive oxygen species to induce differentiation of LSCs. Inhibition of myeloperoxidase activity as well as ROS scavenging – both rescue/block Oral-Aza mediated differentiation of LSCs. Thus, a key understanding from our work is that the impact of oral-Aza is cell type specific.

Azacitidine in combination with other agents has been shown to target LSCs and induce differentiation (37, 38). However, the specific Aza-associated mechanism was not clear. A key understanding from our work is the differential effect of Oral-Aza on LSCs versus non-LSCs. In the in vitro model (OCI-AML20) enriched in LSCs, the extended dose was more effective than the conventional dose. To recapitulate the in vitro findings, we performed engraftment studies of primary AML samples using bulk mononuclear cells which predominantly includes progenitors and blasts. The two dosing regimens performed similarly in reducing disease burden. Notably, conventional Aza is administered for 7 days in the clinic while our animal models received Aza only for 5 days based on our modeling. While we attempted to perform secondary engraftment, a measure of the capacity of re-initiation of leukemia by LSCs when serially transplanted into mice, we did not achieve any engraftment in the conventional or extended-dose regimens even at the site of injection in the secondary mice likely because of the robust effect of both dosing regimens in the primary engraftment (data not shown). Thus, we could not leverage our transplant models to interrogate effects on LSCs in vivo.

Multiple studies have demonstrated that LSCs rely on oxidative phosphorylation, a process that could generate more reactive oxygen species (ROS) and result in loss of stemness (33, 39, 40). However, LSCs have developed unique mechanisms to maintain stemness by countering increased ROS such as increased antioxidant enzymes, increased levels of mitophagy and others (41- 44). Thus, when oxidative stress/damage/signaling increases, stem cells tend to differentiate (45, 46, 33). MPO is a hallmark enzyme of the myeloid lineage and patients with high MPO have been shown to have superior overall survival (47) and has been shown to be expressed at high levels in cells committed to granulomonocytic differentiation and activation of MPO facilitates oxidative damage (48). In addition, MPO has been shown to be increased in response to Aza in a few cases through promoter demethylation by DNMT-inhibitors (49, 50). Our work uniquely demonstrates MPO upregulation in the LSC compartment upon Aza
treatment which induce differentiation through oxidative stress as evidenced by a block in differentiation through ROS scavenging. A limitation of our study is that the cell lines and the LSC model that we have used are not truly representative of de novo AML and do not carry the common molecular mutations observed in elderly AML patients. To this end we attempted to replicate our key findings in commercially available multiple primary AML samples. However, we were successful in culturing CD34+ cells from only one primary sample (TET2 and TP53 mutated and not the commonly observed DNMT3A/NPM1/FLT3 ITD mutation) for 7 days and replicated our key findings.

Overall, we have highlighted mechanistic insights of Oral-Aza that make it an effective maintenance therapy. Aza is known to have additional mechanisms of action beyond the ones described here including immune modulation, viral mimicry, and others. While we have primarily focused on tumor intrinsic mechanisms as part of this work, our data warrant exploring other mechanisms where Aza is implicated and identify further unique properties of Oral-Aza.
REFERENCES


Figure legends

Figure 1. Extended-dose Aza leads to a more sustained hypomethylation and gene expression. (A) Schematic of experimental design (B) OCI-AML2, (C) MV-4-11, and (D) SKM-1 cell lines DNA methylation profiles as measured by whole genome bisulfite sequencing in response to the two dosing regimens. Compared to day 3, in day 5 extended dose results in a progressing/sustained hypomethylation. (E) Barplots indicating the total number of significantly differentially expressed genes in all three cell lines at the two timepoints (day 3 and day 5). Three replicates per cell line, per time point, per gene was used to generate the barplots using the R limma package. Note the increase in the number of genes in extended dose but not in conventional dose Aza at the later timepoint (“down” indicates downregulated and “up” indicates upregulated). (F) DNMT1 levels were measured by protein simple Western at the indicated times in SKM-1 cell line. (G) AUC barplots representing the amount of azacitidine incorporation in PBMCs using the conventional or extended dose of Aza. AUC data was generated from 3 animals and 5 timepoints for each dosing regimen (H) Biochemical analysis was performed in MV-4-11, SKM-1, and AML-193 cells treated with low (0.2 µM) or high (1 µM) dose of Aza for 24 hours. Protein lysates were prepared and assessed for treatment-mediated activation of ISR and loss of DNMT1. AML-193 experiment was duplicated (right panel) to assess the effect of ISRIB, an ISR inhibitor, in ablating Aza-induced ISR activation. Tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (I) AML-193 cells were treated with Aza in conventional or extended regimen and protein lysates were prepared from days 1–7 and assessed for DNMT1 expression and ISR activation markers. EIF2α was used as a loading control.

Figure 2. Extended dosing of Aza preferentially targets bone marrow blasts. (A) Survival of B6 albino mice injected with C1498-Luc3-GFP+ cells and treated with vehicle, conventional-Aza (5 mg/kg QD x 4) or extended-Aza (1 mg/kg QD x 20); n=10 per group. Representative bioluminescence imaging (right panel) of mice are shown (n = 1 representative mouse/group). Blue to red color represents low to high intensity of bioluminescence. (B) Mean spleen weights measured at the end of the study for the PDX AML mice treated with vehicle, conventional-Aza (5 mg/kg QD x 5) or extended-Aza dosing (1 mg/kg QD x 25) are shown as group mean ± SD (n = 5 to 7/group). Red dotted line indicates normal C57BL/6J mouse spleen weight. ** = p ≤ 0.01. (C) Median spleen weights from GEM model of AML harboring FLT3-ITD and TET2 loss (n = 17-19/group) (Normal spleen weight of wild type mice is 72 milligrams). * = p ≤ 0.05; ** = p ≤ 0.01.
0.01, *** = p ≤ 0.001 for azacitidine relative to vehicle/isotype control using nonparametric One-way ANOVA. (D) Flow cytometry was performed on cells collected at the end of week 4 and cell percentages are presented as group medians (n = 12/group). *** = p ≤ 0.001 for azacytidine relative to vehicle/isotype control. P values were calculated using nonparametric One-way ANOVA.

**Figure 3.** Extended dosing of Aza induces greater differentiation of LSCs. (A) OCI-AML20 cells were cultured on OP9 feeder layer and the cells were treated with DMSO or Aza (extended or conventional dosing). At day 7, all cells were collected and subjected to flow cytometry. After discriminating live/dead cells and singlets, human cells (to differentiate from mouse OP9 cells) were identified by CD33+/CD45+ staining. Within that population the profile of CD34/CD38 was identified. (B) CD34+ enriched primary AML sample was treated with both dosing regimens. Flow cytometry was performed on day 7 to identify CD34 and CD38 population. (C) Samples from the above experiment were collected and subjected to scRNA-seq as described in the methods. Violin plots indicating the treatment specific distribution and the Wilcoxon p-value of the GSVA enrichment on Van Galen signatures at days 3, 5, and 7.

**Figure 4.** Extended-dose Aza is equally effective as conventional dosing in targeting AML cells and stem/progenitor cells in vivo. (A) Schematic of experiment. Primary AML cells were injected into the right femurs of sub-lethally irradiated NOD/SCID mice. Twenty days after injection, mice (10 animals per group) were treated with vehicle or two different doses of Aza mimicking conventional/injectable dosing (3 mg/kg 5 doses for 5 consecutive days) or extended dosing (1 mg/kg 15 doses over 21 days (5/7 days per week)). At the end of treatment (42 days from day of injection), flow cytometry was performed in both right and left femurs to measure engraftment. (B) Plots indicate the percentage of human cells that engrafted in the left femur (distal site). The red bar indicates the median values, and each data point indicates a mouse. To measure the ability of Aza to target the persistence of leukemic cells at the site injection, human cells in the right femur (site of injection) were assessed with the indicated surface markers to identify (C) GMP, (D) CMP, and (E) MEP (F) CD123 (IL-3 receptor alpha chain), a marker for AML stem and progenitor cells, was also reduced at the site of injection demonstrating equivalent efficacy for the conventional dosing and the extended dosing of Aza. (G) Animal body weight was monitored during treatment as a proxy for animal health. The dotted line indicates 90% (10% change). Data represent median ± SD. *p < 0.05 and **p < 0.01 by unpaired t test.
Figure 5. Extended-dose Aza upregulates MPO expression to induce LSC differentiation, which can be partly rescued by MPO inhibition or ROS scavenging. (A) OCI-AML20 cells were sorted to retrieve the CD34+/38− population. (B) Sorted cells were treated with Aza (extended or conventional dose) and bulk RNAseq was performed at day 5 followed by pathway enrichment analysis (GO process) to assess Aza induced pathways. (C) OCI-AML20 cells were treated with Aza (conventional or extended regimen) and MPO expression was assessed by capillary electrophoresis on day 7, GAPDH was used as a loading control. (D) Representative flow data from one experiment with OCI-AML20 cells were treated with extended or conventional regimen of Aza with or without 10 µM MPO-IN-28 or (E) N-acetylcysteine (NAC) on days 2 and 4 and flow cytometry assessment was conducted on day 7 using live/dead, CD45, CD34 and CD38 staining.
Figure 2: Extended dosing of Aza preferentially targets bone marrow blasts

Wilcoxon test (n=10/group) p < 0.005 vs Vehicle; Conventional vs Extended dose = NS
Figure 3: Extended-dose Aza induces greater differentiation of leukemic stem cells (LSCs)

A

Day 7

CD38

\(10^1\) \(10^2\) \(10^3\) \(10^4\) \(10^5\) \(10^6\) \(10^7\)

CD34

DMSO
Conventional Aza
Extended Aza

B

CD34+ primary sample

\% of cells

0 20 40 60

CD34+
CD34-

DMSO
Extended Aza
Conventional Aza

C

OCI-AML20 cells: scRNA-Seq

Day 3
Day 5
Day 7

Progenitor

0.5
0
-0.5

p<2.22e-16
p<2.22e-16
p<2.22e-16

0.00019
p<2.22e-16
p<2.22e-16

p<2.22e-16
p<2.22e-16
p<2.22e-16

0.5
0
-0.5

GMP

p<2.22e-16
p<2.22e-16
p<2.22e-16

p<2.22e-16
p<2.22e-16
p<2.22e-16

p<2.22e-16
p<2.22e-16
p<2.22e-16

Myeloid

0.5
0
-0.5

p<2.22e-16
p<2.22e-16
p<2.22e-16

p<2.22e-16
p<2.22e-16
p<2.22e-16

0.55
p<2.22e-16

p<2.22e-16
p<2.22e-16
p<2.22e-16
Figure 4: Extended dose Aza induces differentiation of AML progenitors in vivo

A

Control
Extended dose (1 mg/kg Aza x 15)
Conventional dose (3 mg/kg Aza x 5)

Day 0 20 42

B

Primary engraftment
(CD45+CD33+)

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C

GMP (CD34+ 38+ 123+ 45RA+)

Extended dose
Conventional dose
Control

Percentage of cells

D

CMP (CD34+ 38+ 123+ 45RA-)

Extended dose
Conventional dose
Control

Percentage of cells

E

MEP (CD34+ 38+ 123- 45RA-)

Extended dose
Conventional dose
Control

Percentage of cells

F

Site of injection: CD33+ CD123+

Extended dose
Conventional dose
Control

Percentage of cells

G

Body weight change in animals

% change in body weight

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% change in body weight

20  25  30  35  40  45
Figure 5

A

Input cells
Sorted CD34+/38- cells

CD38

CD34

B

GO Process enriched in extended Aza dosing in CD34+38- cells at day 5

- myeloid leukocyte activation
- regulation of multicellular organismal process
- positive regulation of transport
- leukocyte activation
- regulation of localization
- regulation of response to external stimulus
- regulation of secretion
- regulation of transport
- cellular developmental process
- cell differentiation

C

MPO protein expression

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<th>CD34+ (Primary AML)</th>
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<td>47 KDa Actin</td>
<td>GAPDH</td>
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<td>Conventional</td>
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D

CD38

DMSO
MPO inhibitor

Extended Aza

Conventional Aza

E

DMSO
NAC
Supplemental figure legends

**Figure S1.** Extended-dose Aza leads to a more sustained hypomethylation. (A) OCI-AML2, MV-4-11, and SKM-1 cells treated with extended (blue), or conventional (green) Aza regimen for 5 days were subjected to whole genome bisulfite sequencing to assess genome wide methylation changes. (B) Schematic of dosing mice used for [14C] Aza incorporation. (C and D) Measurements of Aza incorporation using the conventional or extended dose as indicated. The study samples were analyzed for radioactivity in DNA. Results were corrected for background values (5 µl water + 20µl 1.97 mg/ml 14C-paracetamol) and then converted to pgEq Aza using the specific activity of the dose formulation (37 MBq/g) and to pgEQ/µg DNA using the absolute yield of DNA.

**Figure S2.** Bioluminescence imaging results at (A) Day 15, 18 and 21 (B) post injection are presented as group mean ± SEM (n = 10/group). Percentage of C1498-GFP+ cells on Day 25 in the blood.

**Figure S3.** Aza treatment induces differentiation in LSCs. (A) Bar graph of flow cytometry data showing CD34+38- and CD34+38+ cell populations (% of cells) from 3 independent experiments using OCI-AML20 cells with the Aza dosing as indicated. Statistical analysis was performed using two-way ANOVA (B) Flow cytometry assessment, using Annexin V and live/dead stains, was done to assess cell death in OCI-AML20 following Aza treatment, day 7. (C) Primary AML sample harboring TP53 and TET2 mutations were treated with the conventional or extended dose. On day 7, flow cytometry was performed to identify CD34 and CD38 fractions.

**Figure S4.** LogFc of mRNA of secreted factors from LSCs. (A) Bar graph depicting logfc of genes from RNAseq data whose protein secretion changed by more than 20% following Aza treatment, relative to control on days 5 and 7. (B) Bar graphs representing the percentage of progenitor population in OCI-AML20 treated with the indicated Aza dosing with MPO-IN-28 or (C) LSCs treated with NAC on day 7 from three independent experiments. Statistical analysis was performed using two-way ANOVA. * Indicates an adjusted p value of < 0.01
Supplemental methods

Capillary Electrophoresis
Briefly, approximately 5 million cells were lysed with 300–500 µL of cold radioimmunoprecipitation assay (RIPA) buffer containing a protease and phosphatase inhibitor cocktail and vortexed. Samples were then centrifuged at maximum speed at 4°C for 30 minutes. The supernatant was collected, and protein concentration assessed by bicinchoninic acid (BCA) assay. For capillary electrophoresis, 4 µL of 1–1.5 mg/mL of sample was ran in Wess or Jess, according to manufacturer’s protocol, using antibodies to assess specific proteins.

Single Cell RNA-seq
Single-cell suspensions were converted to barcoded single-cell RNA (scRNA)-seq libraries by using the Chromium Next GEM Single Cell 3’ Reagent Kits v3.1 (Dual Index), Gel Bead & Multiplex Kit, and Chip Kit (10x Genomics; Pleasanton, CA), loading an estimated 15,000 cells per library and following the manufacturer’s instructions. The final libraries were profiled using the Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) and sequenced using Nova S4 (Illumina; San Diego, CA) to obtain 10 bp dual index, approximately 200 million reads per library. scRNA-seq data for each sample were processed with CellRanger count (CellRanger 2.1.0; 10x Genomics) using a custom reference package based on mouse and human reference genome, subtracting mouse transcript reads. Subsequent data analysis was carried out in R 3.5.1 and the Seurat package (v2.3.4).

RNA-seq
Control and drug treated cells were lysed by the addition of 350 µL of β-ME containing buffer RLT. Samples were then snap frozen before processing and analysis. Sequencing libraries were created using the IlluminaTruSeq Stranded messenger RNA (mRNA) method, which preferentially selects for messenger RNA by taking advantage of the polyadenylated tail. Libraries were sequenced using the Illumina sequencing-by-synthesis platform, with a sequencing protocol of 100 base-pair (bp) paired-end sequencing and total read depth of 30M reads per sample.

Whole Genome Bisulfite Sequencing
Control or Aza treated cells were pelleted and snap frozen before analysis. DNA extraction from cells was performed with QIAamp DNA kit (Qiagen; Hilden, Germany) and quantified with Qubit 2.0 DNA HS Assay (ThermoFisher; Waltham, MA) followed by quality assessment using Fragment analyzer genomic DNA Assay (Agilent Technologies, Inc.; Santa Clara, CA). Genomic
DNA was bisulfite converted with EZ DNA Methylation-Gold Kit (Zymo Research; Irvine, CA). Library preparation was then performed using Accel-NGS® Methyl-Seq DNA Library kit (Swift Biosciences; Ann Arbor, MI) and quality assessed by TapeStation HSD1000 ScreenTape (Agilent Technologies Inc.). Equimolar pooling of libraries was performed based on QC values. Samples sequencing was done on an Illumina® NovaSeq S4 with a read length configuration of 150PE for 1B PE reads per sample (500M in each direction). A 20% PhiX Spike In was added during the sequencing to ensure sequencing quality.

**Bioinformatic analysis of Whole-Genome Bisulfite Sequencing (WGBS) and RNA-seq**

Bismark methylation counts were restricted to those CpG islands showing at least one methylated residue and at least one unmethylated residue across all samples, and whose total depth was at least seven reads within each sample. Beta values were computed as the methylated residue fraction.

Differences in coverage were assessed by taking the maximum absolute difference between treatment and matched DMSO control and performing 1,000 permutations. The resulting distribution of maximum difference statistics was closely approximated by a gamma distribution; and final p-values were estimated by fitting the parameters of a gamma distribution to the permutated values (per CpG) and computing the distribution survival value of the observed statistic. The resulting distribution of p-values was deflated, and the top hits were on un-localized contigs. We therefore did not control for coverage in downstream analyses.

Regional methylation values for promoters, enhancers, and gene bodies were computed as the total number of methylated reads within an interval, divided by the total number reads. Gene bodies were defined by RefSeq MANE select genes, and promoter regions were defined as 1,000bp upstream of the first base of the gene definition. Enhancer regions were defined by the AML track of EnhancerAtlas (1).

Sample outliers were assessed by taking the principal components of the top 500 most variable CpG sites (by coefficient of variation) and computing the square of the loadings across the top two principal components. Those samples with a final chi-square of 8 or higher were considered as putative outliers and confirmed visually on the loading plots. This process was repeated until no flagged or visually confirmed outliers are present. This process resulted in the exclusion of one sample.

Differential expression was performed using LIMMA with the VOOM transform method, separately within each cell line and treatment date (2). Only the top 12,000 genes (by median expression) were considered for this test. Differential methylation utilized the computed beta values in a linear
model, separately within each cell line and date. TREAT is applied with a log fold change threshold of 1.5 (gene expression) or a beta change of 0.25 (methylation). Gene set over-representation analyses and gene set enrichment analyses were performed using the R package piano using the Stouffer statistic for gene set enrichment analysis (GSEA). Transcription factor enrichment analysis (TFEA) was performed using the TFEA python package. Comparisons between days were performed directly via LIMMA (expression) or a linear model (methylation) using the contrast day 3 to day 5, and comparisons between treatments at a particular timepoint used to contrast conventional versus extended-dose Aza.

**In Vivo Bioluminescence Imaging**
Luciferase activity was measured in animals using an IVIS® SpectrumCT (PerkinElmer, Inc., Waltham, MA). On the day of imaging, animals were injected VivoGlo™ D-Luciferin substrate (Promega Corporations, Madison, WI; Catalog P1043). Sedated animals were used for ventral image acquisition 10 minutes after receiving luciferin substrate injections. Acquired image was analyzed and exported using Living Image software 4.5.1. (PerkinElmer, Inc.). Flux equaling the radiance (photons/s [p/s]) in each pixel summed or integrated over the region of interest area (cm²) x 4π was used to represent tumor burden; the region of interest included the entire animal.

**References:**
Figure S1: Extended-dose Aza leads to a more sustained hypomethylation

A

B
Figure S2: Extended-dose Aza has similar anti-leukemic effect as conventional-Aza.
Supplemental Figure S3: Extended-dose Aza treatment induces differentiation in LSCs

A. Day 7 - OCI-AML20 cells

B. Live/dead stain

C. CD34+ enriched primary AML sample
Supplemental Figure S4: MPO is upregulated in LSCs treated with extended dose Aza

A

Secreted factors from LSCs

LogFc (RNAseq)

B

Progenitor population (%)

DMSO  Extended  Conventional  DMSO  Extended  Conventional

MPO-IN-28

C

LSC population (%)

DMSO  Extended  Conventional  DMSO  Extended  Conventional

NAC
## Supplementary table 1: Comparison of beta values of methylation across cell lines and treatments

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<th>Cell Line</th>
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<th>Ratio of number of CpG sites with Beta&gt; 0.5</th>
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Conventional Aza demethylates DNA more potently than extended Aza at day 3 in OCI-AML2 and MV411. Extended Aza demethylates DNA more potently than conventional Aza at day 3 in SKM1.

Values <1 represent lower methylation in the first term of column "Comparison" (i.e., for DMSO vs conventional, DMSO is first term and Conventional second term)

Values >1 represent higher methylation in the first term of column "Comparison" (i.e., for DMSO vs Conventional, DMSO is first term and Conventional second term)