Biol Blood Marrow Transplant 25 (2019) 1122–1127

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

# Low-Dose Azacitidine with DNMT1 Level Monitoring to Treat Post-Transplantation Acute Myelogenous Leukemia or Myelodysplastic Syndrome Relapse

Masumi Ueda[1,2], Najla El-Jurdi[3,7], Brenda Cooper[3,7], Paolo Caimi[3,7], Linda Baer[3,7], Merle Kolk[3,7], Lauren Brister[3,7], David N. Wald[3,4,7], Folashade Otegbeye[3,7], Hillard M. Lazarus[3,7], Brenda M. Sandmaier[1,2], Basem William[5], Yogen Saunthararajah[6,7], Philip Woost[7], James W. Jacobberger[3,7], Marcos de Lima[3,7,*]

[1] *Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[2] *Division of Medical Oncology, Department of Medicine, University of Washington, Seattle, Washington*
[3] *University Hospitals Seidman Cancer Center and Case Western Reserve University, Cleveland, Ohio*
[4] *Department of Pathology, Case Western Reserve University, Cleveland, Ohio*
[5] *Division of Hematology, Department of Internal Medicine, The Ohio State University Comprehensive Cancer Center, Columbus, Ohio*
[6] *Department of Hematology and Oncology, Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio*
[7] *Case Comprehensive Cancer Center, Cleveland, Ohio*

*Article history:*
Received 30 October 2018
Accepted 21 December 2018

*Key Words:*
Azacitidine
AML
MDS
Allogeneic transplantation
Relapse

A B S T R A C T

Patients with early relapse of acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS) after hematopoietic cell transplantation (HCT) have a poor prognosis, and no standard treatment. Twenty-nine patients with early disease recurrence post-transplantation were treated with azacitidine (AZA; median dose, 40 mg/m$^2$/day for 5 to 7 days). At a median follow-up of 6.3 months (range, 1.3 to 41.1 months), 7 patients (27%) had a response to AZA, defined as complete remission, hematologic improvement, or improved donor chimerism. Response occurred after a median of 3 cycles, and the median duration of response was 70 days (range, 26 to 464 days). Median survival was 6.8 months (95% confidence interval, 3.8 to 11.1 months). Survival was similar in the patients receiving an AZA dose $\leq$40 mg/m$^2$ and those receiving an AZA dose $>$40 mg/m$^2$. Six patients receiving donor lymphocyte infusion with AZA had a response or stable disease without worsening graft-versus-host-disease. We retrospectively used a flow cytometry assay to explore DNA-methyltransferase-1 in blood mononuclear cells as a potential pharmacodynamic marker to assess intracellular drug targeting in 8 patients. No correlation with AZA dose or response was observed. Low-dose AZA appears to have comparable efficacy to higher-dose AZA post-HCT. A significant proportion of this poor-risk population responded to low-dose AZA, suggesting a dose-independent, noncytotoxic mechanism for antileukemic activity.



## INTRODUCTION

Patients with early relapse of myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML) after allogeneic hematopoietic cell transplantation (HCT) have a poor prognosis. The estimated survival rate of patients who relapse within 6 months of HCT is $<$5% to 10% [1]. Although withdrawal of immunosuppression and donor lymphocyte infusion (DLI) are usually used, treatment options are limited owing to residual toxicities of conditioning chemotherapy or myeloablative radiation, as well as the significant risk of graft-versus-host-disease (GVHD). Azacitidine (AZA) is a cytidine analog that incorporates into replicating DNA or RNA, and at doses that do not result in overt cytotoxicity, the incorporated residues bind to DNA-methyltransferase-1 (DNMT1), leading to DNMT1 degradation and subsequent global DNA hypomethylation [2]. Although AZA has activity in the upfront treatment of patients with AML and MDS ineligible for intensive therapies, its use in the post-allogeneic HCT setting may enhance the graft-versus-leukemia effect while also mitigating GVHD [3,4].

Several retrospective studies have reported achievement of complete remission in 10% to 27% of AML patients whose disease relapsed early after HCT and were treated with standard doses of AZA, 75 to 100 mg/m$^2$ daily for 5 to 7 days [5–7]. However, owing to the considerable risk of myelosuppression with

*Financial disclosure*: See Acknowledgments on page 1127.
* Correspondence and reprint requests: Marcos de Lima, MD, Professor of Medicine, University Hospitals Seidman Cancer Center, 11100 Euclid Avenue, Cleveland, OH 44106.
*E-mail address:* marcos.delima@uhhospitals.org (M. de Lima).

https://doi.org/10.1016/j.bbmt.2018.12.764
1083-8791/

any treatment administered early post-transplantation, the use of lower-dose AZA (16 to 40 mg/m$^2$ daily for 5 days) has been investigated previously [8,9]. Optimal dosing of hypomethylating agents is incompletely understood, and lower doses with more frequent administration may achieve better intracellular drug exposure, allowing for optimized DNMT1 depletion [2].

The effects of DNMT1 depletion by AZA are cell cycle S phase-dependent, and only a fraction of malignant cells entering S phase at the time in which intracellular drug concentrations reach levels adequate for DNMT1 depletion will be affected [2]. Intracellular drug exposure is further determined by drug plasma half-life as well as by cytoplasmic enzymes that aid intracellular drug accumulation [2]. Thus, DNMT1 depletion by AZA is dependent on an overlap between cell cycle S phase and adequate intracellular drug concentrations [2]. To investigate this, we developed a multiparametric cell cycle informative flow cytometry DNMT1 assay to measure the direct pharmacodynamic effects of AZA. Here we report clinical response and DNMT1 levels in blood mononuclear cells of patients receiving low-dose AZA for MDS/AML relapse after allogeneic HCT, suggesting that alternative dosing ranges may achieve equal efficacy with less myelosuppression.

## MATERIALS AND METHODS

We retrospectively reviewed 29 patients with AML and MDS who received AZA for treatment of disease relapse after allogeneic HCT at Seidman Cancer Center, Cleveland, Ohio, between January 2009 and August 2016. All were considered ineligible for a second HCT owing to early relapse or poor functional status. A FoundationOne Heme (Cambridge, MA) genomic profiling assay was performed on bone marrow aspirates at the time of relapse. The FoundationOne Heme assay uses DNA and RNA sequencing to detect genomic alterations in >400 cancer-related genes, gene fusions/translocations, and common drivers of hematologic cancers [10]. Donor chimerism was assessed in unfractionated cells from the blood or marrow. Starting dose of AZA was at the discretion of the treating physician. Patient- and disease-specific variables were examined for their effect on survival and response to AZA. Fisher exact and Wilcoxon signed-rank tests were used to compare categorical and continuous variables. Survival was estimated and compared between groups using the Kaplan-Meier method and the log-rank test.

### *Patient Samples*

We obtained cryopreserved blood and marrow mononuclear cells from the institutional biorepository when available. The use of these specimens for research was approved by the Institutional Review Board at University Hospitals Seidman Cancer Center, Cleveland, Ohio. Written informed consent was obtained from each subject before sample collection.

### *Pharmacodynamic Analysis of DNMT1 Flow Cytometry*

Mononuclear cells were isolated from peripheral blood, cryopreserved in 10% dimethyl sulfoxide, and stored in liquid nitrogen. All procedures were performed on ice (except where noted), and all centrifugations were done at 400 × *g* for 5 minutes at 4°C. Samples were rapidly thawed at 37°C and immediately placed on ice. Cells were treated with a final concentration of 1.0% formaldehyde (Polysciences, Warrington, PA) for 10 minutes at room temperature, washed with an excess of ice-cold phosphate buffered saline (PBS), and centrifuged. Cell pellets were resuspended in a small volume (eg, 100 μL) of ice-cold PBS, followed by the addition of 100% methanol ($CH_3OH$) (previously chilled to -80°C) to a final concentration of 90%, incubated on ice for 10 minutes, diluted with an excess of ice-cold PBS, and then centrifuged. Cell pellets were washed once with ice-cold PBS/2% bovine serum albumin (BSA) and centrifuged. To block nonspecific antibody binding, cells were resuspended in ice-cold PBS/2% BSA for 30 minutes. At this point, the cell concentration of each sample was determined using a Hemavet HV950FS Multispecies Hematology System (Drew Scientific, Miami Lakes, FL). Aliquots containing 1 million cells from each sample were placed in 12 × 75-mm tubes and then centrifuged. Cell pellets were resuspended in 100 μL of ice-cold PBS/2% BSA containing unlabeled rabbit anti-Dnmt1 monoclonal antibody (0.0625 μg/test; Abcam, Cambridge, MA; catalog no. ab92314) and incubated for 1 hour. Samples were washed three times, with each wash followed by a 10-minute incubation with 1 mL of PBS/2% BSA and then centrifugation. After the third wash, Cyclin A2-FITC (20 μL/test; clone 11B2G3; Beckman Coulter, Indianapolis, IN; catalog no. A22327), CD45-PerCP (5 μL/test; clone HI30; BioLegend, San Diego, CA; catalog no. 304026), CD117-PE (5 μL/test; clone 104D2D1; Beckman Coulter; catalog no. IM2732U), CD34-PC7 (3 μL/test; clone 581; Beckman Coulter; catalog no. A51077), F(ab′)2-goat anti-rabbit IgG (H+L) Alexa Fluor 647 (0.0938 μg/test; Thermo Fisher Scientific, Waltham, MA; catalog no. A21246) and unlabeled normal mouse IgG (25 μg per test; Thermo Fisher Scientific; catalog no. 10400C) were added in a final volume of 100 μL, followed by incubation for 1 hour. After the incubation and without washing, 3.5 mL of PBS containing 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) was added to each sample. All samples were analyzed on an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific) at a flow rate of 500 μL/minute. Compensation was performed with CompBeads Set Anti-Mouse Ig, κ (BD Biosciences, San Jose, CA; catalog no. 552843) and Flow Cytometry Protein G Antibody Binding Beads (Bangs Laboratories, Fishers, IN; catalog no. 554/11863).

### *Data Analysis*

WinList 3D version 8.0 (Verity Software House, Topsham, ME) was used for postacquisition analysis. Doublet discrimination was performed using the DAPI area and peak signals. Gated singlet events were displayed in bivariate plots of DNMT1 versus DNA content to identify S + G2 + M phase cells as a function of DNA content. The boundaries dividing defining DNMT1-negative events were set as a rectangular region that bisects G0/G1 versus DNMT1 distribution at the mode (peak value) and extends to include all G0/G1 cells below this point. The median DNMT1-negative value was subtracted from the median DNMT1-positive value for S phase cells. This rule-based objective method is based on the idea that DNMT1 is expressed in late G1, S, and G2, which has been determined using HC116 cells and HC116 DKO cells [11] (data not shown). The data were further normalized for antibody-binding bead levels to account for instrument and staining variability of samples stained on different days. DNMT1 levels were calculated from S phase measurements for all peripheral blood mononuclear cells. This allowed for uniform data analysis in patients with and without circulating leukemia cells at the time of blood sampling.

## RESULTS

Patient and transplantation characteristics are summarized in Table 1. The median patient age was 63 years (range, 28 to 74 years). Twelve patients (41.3%) had unfavorable or monosomal karyotype, 17 (58.6%) had measurable residual disease by multiparametric flow cytometry at the time of HCT, and 7 (24.1%) had refractory disease, reflecting an overall high-risk population for relapse after transplantation. A reduced-intensity conditioning regimen was used in 25 patients (86.2%).

Disease relapse occurred at a median of 105 days (range, 23 to 1477 days) after HCT. Relapse characteristics are summarized in Supplementary Tables S1 and S2. Of the 2 patients with isolated central nervous system (CNS) relapse, 1 received prophylactic intrathecal chemotherapy and had no leukemia involvement in the CNS detectable by cytology pretransplantation, and the other received no prophylactic intrathecal chemotherapy and had no CSF assessment before HCT, owing to low clinical suspicion of CNS involvement. One patient experienced isolated extramedullary relapse of thoracic prevertebral myeloid sarcoma masses and received radiation. One patient was started on AZA for decreasing donor chimerism (85%) while in hematologic and molecular remission without measureable residual disease by flow cytometry, but later progressed to marrow relapse of MDS.

The AZA dose was determined by the treating physician. The median starting dose of AZA was 40 mg/m$^2$/day (range, 16 to 100 mg/m$^2$/day) for 5 to 7 days of a 28-day cycle. Subsequent dose increase occurred in 7 patients initially receiving doses of 16 to 50 mg/m$^2$/day with an average maximum increase of 29 mg/m$^2$/day. The dose was subsequently decreased in 2 patients initially receiving doses of 40 and 75 mg/m$^2$/day. The median number of AZA cycles administered was 3 (range 1-19). Ten patients (5 with HLA-matched related donors, 3 with HLA-matched unrelated donors, 2 with HLA-haploidentical donors) received at least 1one donor lymphocyte infusion (DLI) of $1 \times 10^7$ CD3$^+$ cells/kg during AZA treatment (median DLI per patient, 2; range 1-5). The first DLI was given during the first or second cycle of AZA in the majority of patients.

**Table 1**
Patient and Transplantation Characteristics

| Characteristic | Value |
|---|---|
| Age at transplantation, yr, median (range) | 63 (28-74) |
| Sex, n (%) | |
| Female | 10 (34.5) |
| Male | 19 (65.5) |
| Disease, n (%) | |
| AML | 23 (79.3) |
| MDS | 6 (20.7) |
| Secondary AML, n (%) | |
| No | 19 (65.5) |
| Yes | 10 (34.5) |
| Cytogenetics, n (%) | |
| Intermediate | 12 (41.4) |
| Monosomy | 6 (20.7) |
| Unfavorable | 6 (20.7) |
| Unknown | 5 (17.2) |
| Disease status at HCT (AML), n (%) | |
| CR1 | 17 (74.0) |
| CR2 | 3 (13.0) |
| Refractory | 3 (13.0) |
| FLT3 ITD, n (%) | |
| Negative | 14 (48.3) |
| Positive | 5 (17.2) |
| Unknown | 10 (34.5) |
| NPM1 mutation, n (%) | |
| Negative | 11 (37.9) |
| Positive | 4 (13.8) |
| Unknown | 14 (48.3) |
| MRD by flow cytometry pre-HCT, n (%) | |
| No | 12 (41.4) |
| Yes | 17 (58.6) |
| Donor, n (%) | |
| DUCB | 7 (24.1) |
| HLA-haploidentical | 2 ( 6.9) |
| HLA-matched sibling | 8 (27.6) |
| HLA-matched unrelated | 12 (41.4) |
| Graft, n (%) | |
| Mobilized blood | 22 (75.9) |
| Cord blood | 7 (24.1) |
| Preparative regimen, n (%) | |
| Myeloablative | 4 (13.8) |
| Reduced intensity | 25 (86.2) |
| Total body irradiation, n (%) | |
| No | 17 (58.6) |
| Yes | 12 (41.4) |
| ATG, n (%) | |
| No | 16 (55.2) |
| Yes | 13 (44.8) |

MRD indicates measurable residual disease; DUCB, double umbilical cord blood.

At a median follow-up of 6.3 (range 1.3-41.1) months, 7 (27%) had a response to AZA defined as complete remission (CR; 1 patient), hematologic improvement (2 patients), or improvement in donor chimerism (4 patients; median increase, 36%; range, 18% to 59%). Three patients who had isolated CNS or extramedullary relapse were not included in response assessment because they had site-directed therapy (intrathecal chemotherapy or radiation to extramedullary site); they were included in survival analysis only. An additional 6 patients (21%) exhibited stable disease, defined as no increase in circulating blasts for ≥4 weeks. One patient achieved a CR of AML with 7% marrow blasts at relapse, with a sustained CR for >2.5 years without immunosuppression withdrawal due to ongoing chronic GVHD. The median survival for the entire cohort was 6.8 months (95% confidence interval, 3.8 to 11.1 months) (Figure 1A).

In patients who had a response (as defined above), the median number of AZA cycles to the best response was 3 (range, 1 to 4) with a median duration of response of 70 days (range, 26 to 464 days). One patient died of aspergillus pneumonia without disease progression, and 1 patient remained in CR for >2.5 years after initiation of AZA. Any response to AZA (including objective response or stable disease) was associated with increased survival (7.6 months versus 3.2 months; $P = .026$) (Figure 1B). Response (including objective response or stable disease) was significantly higher in those who did not receive antithymocyte globulin (ATG) in the conditioning regimen (77% response rate in the no ATG group versus 23% in the ATG group; $P = .017$). There was no significant difference in response between patients who received DLI and those who did not receive DLI (75% versus 39%; $P = .20$) and between patients who received an AZA dose ≤40 mg/m$^2$ and those who received an AZA dose >40 mg/m$^2$ (63% versus 33%; $P = .23$). Lower-dose or higher-dose AZA was not associated with a significant difference in survival (7.6 months versus 4.9 months; $P = .50$) (Figure 1C).

### *Safety*

Of the 126 total cycles of AZA administered, 49 cycles (39%) were associated with grade 3 or 4 neutropenia or thrombocytopenia. Fourteen patients (48%) had at least 1 cycle delayed for >4 weeks due to infectious complications. Among 16 patients, there were 31 total hospital admissions within 30 days of AZA administration. Only 1 patient died of a cause not attributable to progressive disease. The incidences of grade 3 or 4 neutropenia, grade 3 or 4 infection, number of hospital admissions, inpatient days, and treatment cycle delays were not significantly different between patients receiving an AZA dose of ≤40 mg/m$^2$ and those receiving an AZA dose >40 mg/m$^2$.

### *GVHD*

Twenty-two patients (76%) were receiving systemic immunosuppressive therapy at the time of AZA initiation. Of these, 9 patients had no previous acute or chronic GVHD and were taking a calcineurin inhibitor and/or mycophenolate mofetil for prophylaxis, 10 were receiving immunosuppression for previous acute GVHD, and 3 were receiving immunosuppression for previous chronic GVHD. Of the 22 patients, 11 had immunosuppression successfully tapered or discontinued without subsequent development or recurrence of GVHD. In the entire cohort, 5 patients developed new or recurrent acute GVHD at a median of 76 days (range, 15 to 192 days) after AZA initiation, and all had grade I-II skin GVHD that was successfully treated with systemic or topical steroids; 2 had received DLI as well. Five patients developed mild to moderate chronic GVHD at a median of 114 days (range, 92 to 396 days) after AZA initiation.

### *DNMT1 Assay*

We measured intracellular DNMT1 levels in peripheral blood mononuclear cells in S phase via a multiparametric cell cycle informed flow cytometry assay to explore the pharmacodynamic effects of low-dose AZA (Figure 2). Eight patients had pre-AZA and post-AZA blood samples available (Supplementary Table S3). Pre-AZA baseline samples were obtained within 2 weeks of the initiation of AZA treatment in all but 2 patients in whom pretreatment samples were obtained approximately 8 weeks before AZA initiation. Pretreatment bone marrow samples were not available for any of the subjects. In our limited number of samples, there was no clear association between AZA dose and change in DNMT1 level post-treatment (Figure 3A), although the effect of AZA may become more uniform (less variable) as a function of dose. Five patients displayed a modest change in DNMT1 levels before and after treatment, with more pronounced changes within the first few

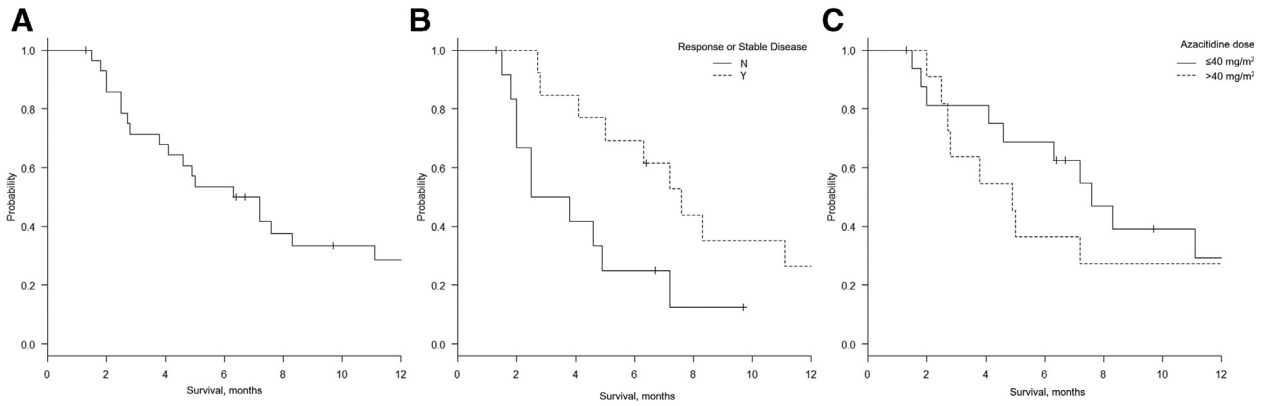


**Figure 1.** Survival from the time of disease relapse after allogeneic HCT. (A) Median survival for the entire cohort was 6.8 months (95% confidence interval, 3.8 to 11.1 months). (B) Survival was significantly higher in those who responded to AZA (7.6 months versus 3.2 months; $P = .026$). Response was defined as complete remission, hematologic improvement, improvement in donor chimerism, or stable disease, defined as no increase in circulating blasts for ≥4 weeks. (C) Survival did not differ significantly between patients receiving lower AZA doses and those receiving higher AZA doses (7.6 months versus 4.9 months; $P = .50$).

days of a treatment cycle (Figure 3B). Finally, patients who had an objective response to treatment exhibited lower DNMT1 levels at baseline, as well as a smaller fraction of blood cells in S phase (Figure 3C).

## DISCUSSION

In our analysis of patients with AML and MDS with early disease relapse after allogeneic transplantation who were not eligible for more aggressive therapies, treatment with low-dose AZA with or without DLI showed a durable response in a proportion of patients. One patient who experienced full hematologic relapse achieved long-term CR.

AZA has been shown to promote the graft-versus-leukemia effect through enhanced expression of leukemia-associated antigens [12]. Increases in leukemia-directed cytotoxic T cells also have been observed in patients after AZA treatment [3]. Corresponding to this observation, we found lower response rates in patients who received ATG in the conditioning regimen. Although this finding needs to be confirmed in a larger cohort, it suggests that depletion of donor T cells by ATG may reduce the potential for AZA to enhance donor T cell-mediated antileukemic effects. On the other hand, AZA has been shown to enhance the proliferation of T regulatory cells [3], possibly resulting in mitigation of GVHD. Murine models and human studies have shown inhibition of T cell proliferation and decreased production of proinflammatory cytokines in an apoptosis-independent manner after AZA administration. In addition, reexpression of *FOXP3* and expansion of regulatory T cells after drug exposure has been observed [4]. Thus, the immunomodulatory role of AZA in the post-transplantation setting may have implications in disease control as well as in control of GVHD.

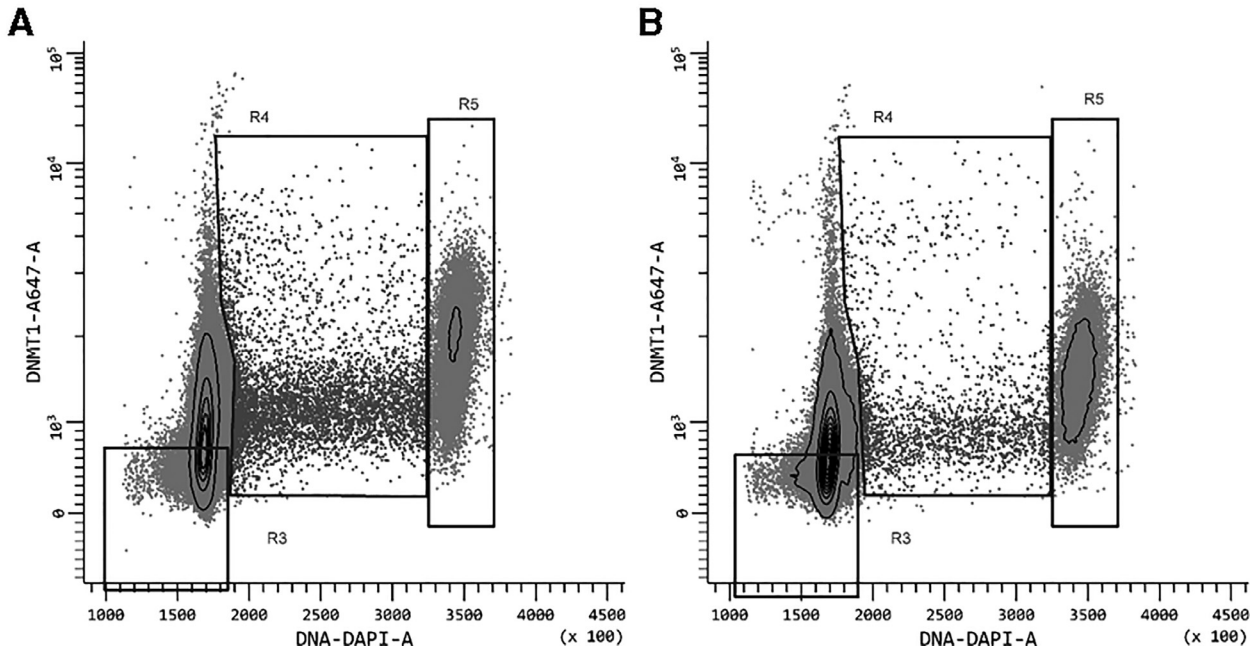


**Figure 2.** Intracellular DNMT1 measured by flow cytometry in peripheral blood mononuclear cells in patient 11 before (A) and 3 days after initiation (B) of daily azacitidine administration. After doublet discrimination was performed using the DAPI area and peak signals, gated singlet events were displayed by DNA content to identify S (R4) and G2 + M (R5) phase cells. DNMT1 was measured as the median DNMT1 negative value subtracted from the median DNMT1-positive value for S phase cells.

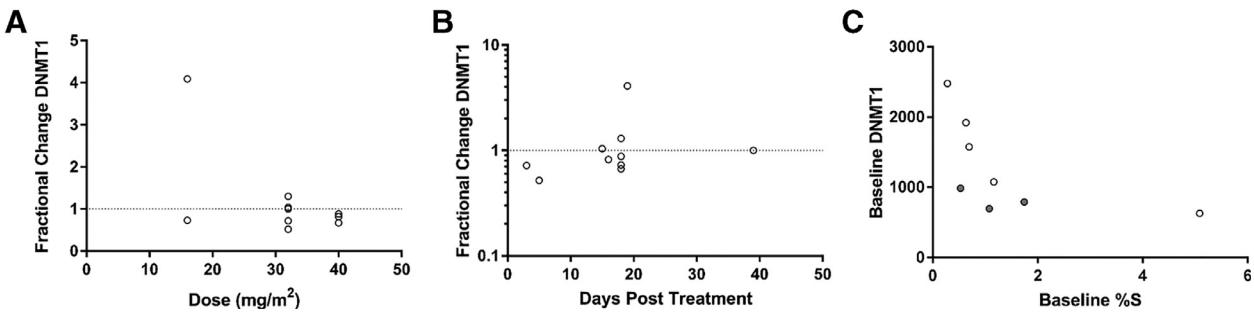


**Figure 3.** Post-treatment DNMT1 levels as a fraction of pretreatment baseline measured by flow cytometry. Samples were available for 8 patients at 10 post-treatment time points. (A) AZA dose and change in DNMT1 level post-treatment. (B) Change in DNMT1 level relative to day of treatment cycle. (C) Baseline DNMT1 levels and percentage of peripheral blood mononuclear cells in S phase pretreatment. Dark circles represent patients who exhibited a response to treatment. DNMT1 was measured as the median DNMT1-negative value subtracted from the median DNMT1-positive value for S phase cells.

Genomic profiles at the time of relapse was available for 7 patients (Supplementary Table S2). In previous studies, *TET2* and *DNMT3A* mutations were associated with a response to hypomethylating agents in the nontransplantation setting [13] and the post-transplantation setting [14], whereas *TP53* mutations were associated with a poor response to post-transplantation azacitidine [14]. In our cohort, 2 patients had a *TET2* mutation at the time of relapse, 1 of whom exhibited stable disease on azacitidine therapy. Although our study is limited by a small sample size, genomic profiling and mutational analysis post-transplantation may be used in the future to inform which patients will benefit from hypomethylating agents for treatment or prevention of relapse.

The occurrence of new acute GVHD after AZA treatment in our cohort was limited to grade I or II skin GVHD responsive to systemic or topical steroids in all cases. Furthermore, most patients who were receiving immunosuppressive therapy at the time of AZA initiation were tapered successfully. Notably, only 2 of the 10 patients in our cohort who received DLI experienced an acute GVHD flare. Consistent with this finding, other studies have reported low rates of GVHD with prophylactic use of concurrent AZA and DLI to prevent relapse after transplantation [15].

Although hypomethylating agents are typically considered noncytotoxic therapy, cytopenias are a major reason for infectious complications, bleeding, and delay or discontinuation of therapy [2]. Myelosuppression is a major concern, especially in the early post-transplantation setting. Previous retrospective reports of treatment of relapsed AML or MDS after transplantation with standard doses of AZA report an approximate 65% incidence of grade 3 or 4 cytopenia [7]. Although many factors, particularly baseline myelosuppression related to the underlying disease or recent conditioning chemotherapy, confound this analysis, the 39% incidence of grade 3 or 4 neutropenia or thrombocytopenia in our cohort is lower than cited in previous reports. Lower AZA doses may explain this difference, although such a conclusion requires confirmation in a larger study.

Lower doses of AZA have been proposed in both the upfront treatment of MDS and AML and the post-transplantation setting, in part to avoid the myelosuppression associated with unwanted side effects. In 1 study, 5 of 9 patients (55%) with recurrent AML at a median of 8 months after HCT who received AZA 16 to 40 mg/m$^2$ daily for 5 days had a response, of whom 3 achieved complete remission for up to 15 months [9]. Low-dose AZA has also been demonstrated to be safe for use as maintenance therapy after allogeneic transplant, with a daily dose of 32 mg/m$^2$ for 5 days of 28-day cycles for 4 cycles found to be the most optimal in terms of time to development of toxicity [8]. Evidence of response to this agent at low doses further support its investigation in the preventive setting. Maintenance oral azacitidine (CC-486) after HCT in patients with AML and MDS may allow for extended dosing and prolonged drug exposure, and a prospective dose-finding study recently showed this agent to be well tolerated with low rates of relapse, disease progression, and GVHD [16].

There is no standard of care for treating relapsed MDS or AML after allogeneic HCT. As with the patients in our cohort, those who experience early disease relapse are often not candidates for intensive therapy or a second allogeneic HCT, and outcomes are poor. A previous large retrospective analyses found an estimated 2- to 3-year survival of 4% to 9% in patients with relapsed disease at <180 to 200 days post-transplantation [1]. In a prospective study of 39 patients with early relapse (<100 days) or persistent AML/MDS after allogeneic HCT, Woo et al [17] found a 2-year overall survival of 25% with the use of AZA 75 mg/m$^2$. The patients in our cohort were not candidates for more intensive approaches to treatment, and the observed response and survival values are comparable to those obtained with more intensive chemotherapy. For patients who achieve CR or a sustained response to AZA, a second allogeneic HCT is a consideration. In our cohort, 1 patient went on to a second allogeneic HCT, a rate comparable to the 2% reported in other retrospective analyses of early post-transplantation relapse [1].

Finally, we retrospectively tested a multiparametric cell cycle informed flow cytometry assay to measure DNMT1 before and after AZA treatment in a limited number of patients. Although conclusions cannot be drawn from the data in this small cohort, lower pretreatment DNMT1 expression may be associated with response, as was seen in our longest-responding patient who achieved CR of overt marrow relapse. Furthermore, owing to the retrospective nature of the study, the timing of sample collection was not predetermined, and most samples were obtained at 2 to 3 weeks after a cycle of drug administration. A more significant degree of DNMT1 depletion may be captured when blood samples are obtained within a short period (hours to days) after drug exposure rather than many days after drug administration, given the short plasma half-life of AZA. These studies are currently ongoing. Although such pharmacodynamic monitoring would be useful in all settings, it is particularly important in the post-transplantation setting, in which myelosuppression and additional toxicity are of even greater concern.

In conclusion, low-dose AZA may be effective in a small proportion of patients with high-risk MDS and AML with early relapsed disease after allogeneic HCT. It appears to cause less cytopenia and does not exacerbate GVHD. Pharmacodynamic

monitoring as a strategy to optimize AZA use merits prospective assessment. Low-dose AZA provides a platform for other immune manipulations to prevent relapse after allogeneic HCT, such as immune checkpoint inhibitors or other strategies that would capitalize on increased tumor immunogenicity.

## ACKNOWLEDGMENTS

*Financial disclosure:* This work was funded in part by a Conquer Cancer Foundation of the American Society of Clinical Oncology Young Investigator Award YIA 9939 (to M.U.) and American Cancer Society Grant IRG-91-022-18 (to B.M.W.). *Any opinions, findings, and conclusions expressed in this manuscript are those of the authors and do not necessarily reflect those of the American Society of Clinical Oncology or the Conquer Cancer Foundation.*

*Conflict of interest statement: There are no conflicts of interest to report.*

## SUPPLEMENTARY MATERIALS

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.12.764.

## REFERENCES

1. Mielcarek M, Storer BE, Flowers ME, Storb R, Sandmaier BM, Martin PJ. Outcomes among patients with recurrent high-risk hematologic malignancies after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2007;13:1160–1168.
2. Sauntharajah Y. Key clinical observations after 5-azacytidine and decitabine treatment of myelodysplastic syndromes suggest practical solutions for better outcomes. *Hematology Am Soc Hematol Educ Program*. 2013;2013:511–521.
3. Goodyear OC, Dennis M, Jilani NY, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood*. 2012;119:3361–3369.
4. Sánchez-Abarca LI, Gutierrez-Cosio S, Santamaria C, et al. Immunomodulatory effect of 5-azacytidine (5-azaC): potential role in the transplantation setting. *Blood*. 2010;115:107–121.
5. Tessoulin B, Delaunay J, Chevallier P, et al. Azacitidine salvage therapy for relapse of myeloid malignancies following allogeneic hematopoietic SCT. *Bone Marrow Transplant*. 2014;49:567–571.
6. Steinmann J, Bertz H, Wasch R, et al. 5-Azacytidine and DLI can induce long-term remissions in AML patients relapsed after allograft. *Bone Marrow Transplant*. 2015;50:690–695.
7. Schroeder T, Rachlis E, Bug G, et al. Treatment of acute myeloid leukemia or myelodysplastic syndrome relapse after allogeneic stem cell transplantation with azacitidine and donor lymphocyte infusions: a retrospective multicenter analysis from the German Cooperative Transplant Study Group. *Biol Blood Marrow Transplant*. 2015;21:653–660.
8. de Lima M, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. *Cancer*. 2010;116:5420–5431.
9. Jabbour E, Giralt S, Kantarjian H, et al. Low-dose azacitidine after allogeneic stem cell transplantation for acute leukemia. *Cancer*. 2009;115:1899–1905.
10. Foundation Medicine, Inc. FoundationOne®Heme. Available at: https://www.foundationmedicine.com/genomic-testing/foundation-one-heme#overview. Accessed April 15, 2018.
11. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*. 2002;416:552–556.
12. Almstedt M, Blagitko-Dorfs N, Duque-Afonso J, et al. The DNA demethylating agent 5-aza-2′-deoxycytidine induces expression of NY-ESO-1 and other cancer/testis antigens in myeloid leukemia cells. *Leuk Res*. 2010;34:899–905.
13. Coombs CC, Sallman DA, Devlin SM, et al. Mutational correlates of response to hypomethylating agent therapy in acute myeloid leukemia. *Haematologica*. 2016;101:e457–e460.
14. Woo J, Howard NP, Storer BE, et al. Mutational analysis in serial marrow samples during azacitidine treatment in patients with post-transplant relapse of acute myeloid leukemia or myelodysplastic syndromes. *Haematologica*. 2017;102:e216–e218.
15. Guillaume T, Yakoub-Agha I, Tabrizi R, et al. Prospective phase II study of prophylactic azacitidine and donor lymphocyte infusions following allogeneic hematopoietic stem cell transplantation for high-risk acute myeloid leukemia and myelodysplastic syndrome. *Blood*. 2016;128:1162.
16. de Lima M, Oran B, Champlin RE, et al. CC-486 maintenance after stem cell transplantation in patients with acute myeloid leukemia or myelodysplastic syndromes. *Biol Blood Marrow Transplant*. 2018;24:2017–2024.
17. Woo J, Deeg HJ, Storer B, et al. Factors determining responses to azacitidine in patients with myelodysplastic syndromes and acute myeloid leukemia with early post-transplantation relapse: a prospective trial. *Biol Blood Marrow Transplant*. 2017;23:176–179.