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Maintenance Therapy with Decitabine after Allogeneic Stem Cell Transplantation for Acute Myelogenous Leukemia and Myelodysplastic Syndrome

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A B S T R A C T

Decitabine is a hypomethylating agent that irreversibly inhibits DNA methyltransferase I, inducing leukemic differentiation and re-expression of epigenetically silenced putative tumor antigens. We assessed safety and efficacy of decitabine maintenance after allogeneic transplantation for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Decitabine maintenance may help eradicate minimal residual disease, decrease the incidence of graft-versus-host disease (GVHD), and facilitate a graft-versus-leukemia effect by enhancing the effect of T regulatory lymphocytes. Patients with AML/MDS in complete remission (CR) after allotransplantation started decitabine between day +50 and +100. We investigated 4 decitabine doses in cohorts of 4 patients: 5, 7.5, 10, and 15 mg/m^2/day × 5 days every 6 weeks, for a maximum 8 cycles. The maximum tolerated dose (MTD) was defined as the maximum dose at which ≤ 25% of people experience dose-limiting toxicities during the first cycle of treatment. Twenty-four patients were enrolled and 22 were evaluable. All 4 dose levels were completed and no MTD was reached. Overall, decitabine maintenance was well tolerated. Grade 3 and 4 hematological toxicities were experienced by 75% of patients, including all patients treated at the highest dose level. Nine patients completed all 8 cycles and 8 of them remain in CR. Nine patients died from relapse (n = 4), infectious complications (n = 3), and GVHD (n = 2). Most occurrences of acute GVHD were mild and resolved without interruption of treatment; 1 patient died of acute gut GVHD. Decitabine maintenance did not clearly impact the rate of chronic GVHD. Although there was a trend of increased FOXP3 expression, results were not statistically significant. In conclusion, decitabine maintenance is associated with acceptable toxicities when given in the post-allotransplantation setting. Although the MTD was not reached, the dose of 10 mg/m^2 for 5 days every 6 weeks appeared to be the optimal dose rather than 15 mg/m^2, where most hematological toxicities occurred.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is a potentially curative therapy for patients with high-risk acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).

Over the past decade, we have witnessed significant advances in therapeutic approaches for alloHSCT, including the use of alternative stem cell sources, less toxic conditioning regimens, and better supportive care, resulting in improved overall survival (OS) [1-5]. However, disease relapse remains the principal cause of treatment failure for these patients [6-9]. The risk of relapse varies from 20% to 60%, depending on the diagnosis and stage of the disease at the time of transplantation, and outcomes of salvage treatments are poor [10-12]. Median time to relapse after nonmyeloablative alloHSCT is 3 to 6 months and somewhat longer after myeloablative alloHSCT. Therefore, early maintenance therapy, directed at eliminating minimal residual disease and promoting a graft-versus-leukemia (GVL) effect, could be an effective method to improve outcomes after alloHSCT.

The concept of post-transplantation maintenance therapy with hypomethylating agents in AML and MDS has been

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initially studied by investigators at MD Anderson Cancer Center [13-15]. They showed that azacitidine (AZA) can be safely administered to heavily pretreated post-transplantation patients and may prolong event-free and OS. In addition, recent studies have shown that AZA, followed by donor lymphocyte infusion, is well tolerated when administered for early AML/MDS relapse after alloHSCT [16-18]. Decitabine (5-aza-2′-deoxycytidine) is a hypomethylating agent that irreversibly inhibits DNA methyltransferase I (DNMT-1), leading to genome-wide global DNA hypomethylation. Although AZA incorporates primarily into RNA and to the lesser extent DNA, decitabine is more selective, reducing only DNA methylation. In addition, decitabine is an approximately 5-fold more potent inhibitor of DNMT-1 than AZA is. Decitabine induces leukemic differentiation and re-expression of tumor-associated genes that had been epigenetically silenced [19]. At high doses, cells die from apoptosis triggered by DNA synthesis arrest, and at low doses, cells survive but change their gene expression profile to favor differentiation, reduced proliferation, and increased apoptosis. In addition, maximum effects of DNA hypomethylation have been observed at low doses and with less side-effects [20,21]. Decitabine has demonstrated activity in a variety of hematological malignances, including AML, MDS, and blast phase chronic myeloid leukemia [22-30]. It is generally well tolerated, with the primary toxicity being prolonged myelosuppression. Our group has demonstrated that decitabine enhances FOXP3 expression and can convert $CD4^+CD25^-FOXP3^-$ T cells into $CD4^+CD25^+FOXP3^+$ T regulatory cells (Tregs) [31,32]. Through their immunoregulatory properties Tregs are thought to play an important role in modulating graft-versus-host disease (GVHD) without sacrificing the beneficial GVL effect [33,34]. In addition, several other groups have demonstrated effects of DNA hypomethylating agents on T cell–mediated antitumor activity [35-39]. These include increasing tumor-specific CD8 T cell responses by upregulating tumor antigen expression on malignant cells and inducing expression of killer cell immunoglobulin–like receptor in T cells, thereby enhancing cytotoxic effector function of T cells against tumors. In human studies, patients noted to have a higher relative frequency of Tregs after HSCT had lower rate and severity of GVHD, lower rate of nonrelapse mortality (NRM), and equivalent relapse mortality [34].

Taken together, these studies provide a rationale for the administration of decitabine after alloHSCT for AML and MDS. We hypothesized that decitabine maintenance may provide direct antileukemic effect both to eradicate minimal residual disease and provide disease control by facilitating a GVL effect. In addition, decitabine may decrease the incidence of GVHD by enhancing the effect of Treg lymphocytes.

PATIENTS AND METHODS

This was a single-institution, open-label, prospective, dose-finding study of low-dose decitabine as a maintenance therapy after alloHSCT. The trial was approved by the institutional review board at the Washington University School of Medicine and informed consent was obtained in accordance with the Declaration of Helsinki. The trial was registered at www.clinicaltrials.gov (NCT00986804). Eisai Inc. provided decitabine for all enrolled patients.

Eligibility Criteria and Enrollment

Adults 18 years of age or older, with AML or MDS, who achieved a complete remission (CR) after alloHSCT were enrolled in the study between day +50 and +100 after alloHSCT. Exceptions were made for 3 patients who were enrolled shortly after day +100: for 2 patients, day +100 fell during the weekend/holiday, and for 1 patient, who was already consented, lived far away, and had transportation problems. *CR* was defined as $< 5\%$ blasts in the bone marrow with a count of at least 200 nucleated cells, no blasts with Auer rods, no extramedullary disease, absolute neutrophil count (ANC) $\geq 1500/\mu L$, platelet count $\geq 50{,}000/\mu L$, and no blasts in the peripheral blood. The higher threshold for ANC than that recommended by International Working Group (IWG), was chosen in anticipation of neutropenia secondary to decitabine and lower platelet threshold was allowed to facilitate enrollment. No platelet transfusion within 7 days of enrollment or growth factor support was allowed to meet those criteria. Other major eligibility criteria included Eastern Cooperative Oncology Group performance status ≤ 2, no grade 3 and 4 acute GVHD, no uncontrolled infection, creatinine $< 1.5 \times$ upper limit of normal (ULN), bilirubin $\leq 1.5 \times$ ULN, and hepatic enzymes $\leq 2.5 \times$ ULN. Both myeloablative and nonmyeloablative conditioning regimens were allowed, and both related and unrelated donors were permitted using either peripheral blood or bone marrow as a source of graft. Donors could be mismatched at a single antigen at HLA-A, -B, or -DR locus, plus a single antigen mismatch at HLA-C; 2-antigen mismatch at a single locus was not allowed. Acute GVHD prophylaxis was according to the treating physician.

Treatment Plan

Decitabine was administered as an intravenous infusion for 5 consecutive days every 6 weeks for up to 8 cycles. The study consisted of 5 escalating dose levels, only 1 of which was open for accrual at a given time. The first cohort of 4 patients started decitabine at 5 mg/m^2/day. In subsequent cohorts, the dose was escalated to 7.5, 10, and 15 mg/m^2/day according to the dose-limiting toxicity (DLT) experienced at the previous dose level. Decitabine dose could be de-escalated to 2.5 mg/m^2/day if a DLT were observed in the first cohort. There was an observation period of 42 days between enrolling each subsequent cohort.

The primary goal was to determine the *maximum tolerated dose* (MTD) of decitabine, defined as the maximum dose at which $\leq 25\%$ of patients experience DLT during the first cycle of treatment. *DLT* was defined as (1) ANC $< 500/\mu L$ and/or platelet count $< 30{,}000/\mu L$ sustained for 2 consecutive weeks without platelet transfusions, (2) inability to achieve ANC $\geq 1000/\mu L$ and platelet count $\geq 50{,}000/\mu L$ after a delay of the second cycle by a maximum 2 weeks, or (3) grade 3 and 4 nonhematological toxicities related to decitabine. Patients who met criteria for DLT started the second cycle at 1 level dose reduction. Patients with inadequate counts 6 weeks after a previous cycle had their next cycle delayed by maximum of 2 weeks. Each cohort could contain 4 or 8 evaluable patients until a MTD or a dose of 15 mg/m^2/day were reached. Patients with documented progressive disease were removed from the study.

Dose Adjustments

Dose adjustments were made as follows: If no DLT were observed in any of the 4 patients treated at the current dose-level, the current dose was deemed acceptable and the study proceeded with dose escalation. If a DLT were observed in 1 of 4 patients treated at current dose-level, then 4 additional patients were enrolled at same dose-level. If 1 of those 8 experienced a DLT, then the current dose was deemed acceptable and escalated; if 2 or more of 8 patients experienced DLTs, then the current dose was deemed over toxicity and the previous dose was considered MTD. If 2 or more of 4 patients experienced a DLT, the current dose was deemed over toxicity and the previous dose level was considered MTD.

Evaluation of Response

All patients completing the first cycle of decitabine were included in the safety and efficacy assessment. History, physical exam, complete blood count, and complete metabolic panel were performed at baseline and every 2 weeks thereafter. Bone marrow biopsy was performed at baseline, at cycle 3 day 1, and at cycle 8 day 42 (or at end of study for any reason). Acute and chronic GVHD evaluation were performed every 2 cycles or sooner, if clinically indicated. Chronic GVHD was diagnosed and graded according to the National Institutes of Health Criteria [40]. Determination of relapse was based on the peripheral blood, bone marrow biopsy, or evidence of new extramedullary disease. Patients were followed for survival and relapse for 5 years.

Toxicity Assessment

All patients receiving at least 1 dose of decitabine were included in the toxicity assessments. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria, version 3.0. *Serious adverse event* was defined as any toxicity that met any of the following conditions: resulted in death, was life threatening, required hospitalization, or resulted in significant disability or incapacity. Toxicity assessment was performed at the beginning of each cycle.

Table 1
Patients' Characteristics

Cohort (Dose)	Patient No.	Sex/Age, y	Diagnosis and Status at alloHSCT	Disease Risk	Donor Type	Conditioning Regimen	Days from alloHSCT to Dec	Chimerism at Enrollment, %
1 (5 mg/m^2)	1	M/62	AML-PD	Ph+ AML	mMUD	BU/CY	95	100
	2	F/59	AML-PD	Prior MF, trisomy 8, FLT3-ITD+	MRD	BU/CY	62	Mixed
	3	M/37	AML-CR1	Monosomy 7	MUD	BU/CY	75	100
	4	M/62	AML-PD	Prior MDS, trisomy 19	MUD	TBI/CY	95	Mixed
	5	M/57	MDS	Trisomy 8, 5q-	MUD	BU/CY	76	100
	6	M/59	AML-CR2	t (8,21), CR2	MUD	BU/CY	77	100
	7	M/42	AML-CR1	Del 12/ETV6	mMUD	BU/CY	115	100
	8	M/52	MDS	Del 5, Del 6	MUD	BU/CY	90	100
	9	M/58	AML-CR1	Complex cytogenetics	MRD	BU/CY	98	100
2 (7.5 mg/m^2)	10	F/68	AML-CR1	Complex cytogenetics	MRD	FLU/BU	96	100
	11	F/65	AML-CR1	FLT 3-ITD +	MUD	TBI/CY	96	100
	12	F/45	AML-CR1	Therapy-related AML	mMRD	BU/CY	98	100
	13	M/63	AML-CR1	Prior MDS	mMUD	BU/CY	103	100
3 (10 mg/m^2)	14	M/21	AML-CR1	FLT3-ITD +	MUD	BU/CY	97	100
	15	F/56	AML-CR1	Complex cytogenetics	MUD	BU/CY	87	100
	16	M/62	MDS	High-grade MDS	MUD	BU/CY	95	100
	17	M/63	AML-CR1	Prior MDS	MRD	BU/CY	102	100
4 (15 mg/m^2)	18	F/54	AML-CR2	CR2	MRD	BU/CY	95	100
	19	F/51	AML-CR1	MLL rearrangement	MUD	BU/CY	82	100
	20	M/57	MDS	Del 7	mMUD	BU/CY	86	100
	21	M/67	AML-CR1	Del 17p (p53)	MUD	FLU/BU	74	100
	22	M/66	MDS	High-grade MDS	MRD	BU/CY	100	100

Dec indicates decitabine; M, male, PD, persistent disease; Ph, Philadelphia chromosome; mMUD, mismatched unrelated donor; BU, busulfan; CY, cyclophosphamide; F, female; MF, myelofibrosis; MRD, matched related donor; MUD, matched unrelated donor; TBI, total body irradiation; Del, deletion; Flu, fludarabine; mMRD, mismatched related donor; FLT3, FMS-like tyrosine kinase 3; MLL, mixed-lineage leukemia gene translocation.

Correlative Studies

Peripheral blood samples to examine levels of lymphocyte populations were obtained at baseline, immediately before administration of decitabine on cycle 1 day 1 (C1D1) and cycle 3 day 1, immediately after administration of decitabine on cycle 1 day 5 (C1D5) and cycle 3 day 5, and at the end of study (EOS). Flow cytometry of peripheral blood was performed to examine lymphocyte populations including $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+FOXP3^+$ Tregs, $CD3^-CD19^+$ B cells, and $CD3^-CD56^+$ natural killer (NK) cells. The following antibodies were used: CD3 PerCP-Cy5.5 (OKT3, eBioscience, San Diego, CA), CD4 PE (RPA-T4, BD Biosciences, Franklin Lakes, NJ), CD4 APC-eFluor780 (RPA-T4, eBiosciences), CD8 APC (RPA-T8, BD Biosciences), CD19 APC (HIB19, BD Biosciences), CD56 PE (B159, BD Biosciences), CD45 FITC (HI30, BD Biosciences), and FOXP3 PE (236A/E7, eBiosciences). To determine absolute numbers of these cells, Sphero Accucount Fluorescent Particles (Spherotech, Lake Forest, IL) were used. To measure DNA methylation in the FOXP3 locus, $CD4^+$ T cells were isolated from the cohort treated with the highest dose of decitabine (15 mg/m^2/day) using a Sony SY3200 cell sorter (Sony Biotechnology, San Jose, CA) operated by the Siteman Cancer Center Flow Cytometry shared resource. The purities of the cells were 90% or higher. Direct bisulfite modification from cells and pyrosequencing analysis were performed by EpigenDx (Hopkinton, MA). A total of 16 CpGs were analyzed: 5 CpGs in 5′ UTR region (−6278 to −6216 from ATG, Ensembl Transcript ID ENST00000376207), 2 CpGs in the human Treg specific demethylated region (TSDR) (−2376 to −2371 from ATG), and 9 CpGs in the TSDR (−2330 to −2263 from ATG) [41-43].

Statistical Analysis

Demographic and clinical characteristics of patients, as well as outcomes and length of follow-up, were listed for each patient. Preliminary efficacy was also assessed from pooled data of all cohorts. The endpoints for efficacy included relapse of the hematological malignancy, incidence and severity of GVHD, as well as OS and disease-free survival (DFS). *OS* was defined as the time from transplantation to death from any cause, and survivors were censored at the date of last contact. *DFS* was defined as time from transplantation to relapse or death, whichever occurred first. Those patients alive and relapse free were censored at date of last contact. Probabilities of OS and DFS were estimated using the Kaplan-Meier product-limit method. The cumulative incidence of relapse was estimated using Gray's subdistribution method to account for the presence of competing risk because of NRM [44]. Statistical analyses were performed using statistical packages cmprsk (http://biowww.dfci.harvard.edu/~gray) for competing risk analysis and SAS 9.2 (SAS Institutes, Cary, NC) for all other analyses.

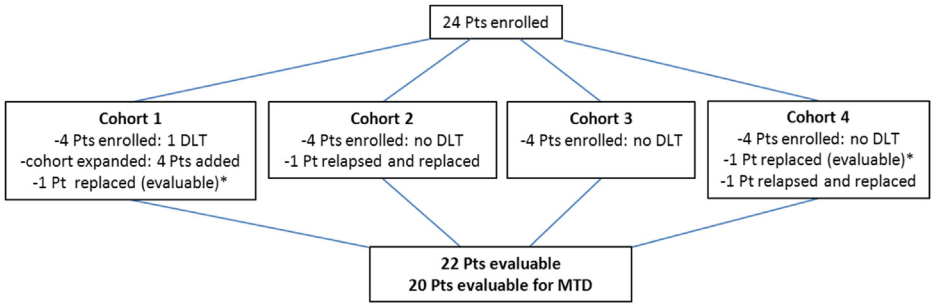
RESULTS

Patient Characteristics

Between May 2010 and April 2014, 24 patients were enrolled and treated on this study. Two patients failed to complete the first cycle of treatment because of early relapse; they were replaced and excluded from all analyses other than toxicity. The characteristics of 22 evaluable patients are shown in Table 1. There were 15 males. Median age was 59 years (range, 21 to 68). Seventeen patients had AML and 5 had MDS. Twenty patients received myeloablative conditioning (2 total body irradiation–based conditioning) and 2 patients received nonmyeloablative conditioning. Six patients received alloHSCT from 10/10 HLA–matched siblings, 1 from 8/10-mismatched sibling, 11 from 10/10-matched unrelated donors, and 4 from mismatched unrelated donors. All patients received mobilized peripheral blood stem cells as the graft source and methotrexate and tacrolimus as GVHD prophylaxis. All patients were in CR at the time of enrollment. The median time from alloHSCT to start of decitabine was 95 days (range, 62 to 115).

Dosing of Decitabine

Four patients were initially enrolled in cohort 1 (5 mg/m^2/day) (Figure 1). One patient experienced a hematological DLT and, therefore, 4 additional patients were added to expand that cohort. None of the additional patients experienced a DLT. One patient was replaced as he failed to receive all cycle 1 doses because of noncompliance; however, this patient received subsequent cycles and remains evaluable for all assessments besides MTD. Four patients were enrolled in cohort 2 (7.5 mg/m^2/day). One patient relapsed before completing cycle 1 and was replaced. No DLTs were observed in that cohort; therefore, 4 patients were enrolled in cohort 3 (10 mg/m^2/day). None of those patients experienced a DLT and 4 patients were enrolled in cohort 4 (15 mg/m^2/day). There were no DLTs at this dose level; however, 2 patients



*Evaluable for all the assessments except MTD

Abbreviations: DLT-dose limmiting toxicity; MTD- maximal tolerated dose; Pts- patients

Figure 1. Cohorts' characteristics.

were replaced. One patient was replaced because of the early relapse, before completing cycle 1. The other replaced patient was deemed nonevaluable for DLT because he missed several laboratory data points and received growth factors for neutropenia during the first cycle; his decitabine dose was reduced to 10 mg/m^2/day and he completed the remaining cycles at that dose. In summary, as only 1 patient met the criteria for DLT, the MTD was not established.

Response and Mortality

The median number of cycles completed was 5 (range, 1 to 8). The causes for early discontinuation were: toxicity (n = 5), relapse (n = 3), new-onset severe gut GVHD (n = 1), physician discretion (n = 1), noncompliance (n = 2), and consent withdrawal (n = 1) (Table 2). Nine of 22 patients (41%) completed 8 cycles of decitabine and all of them are alive: 8 patients are in CR and 1 patient developed central nervous system (CNS) relapse and leukemia cutis 1 year after completing decitabine maintenance and underwent a second alloHSCT. The remaining 13 patients discontinued decitabine before cycle 8 and only 4 are alive: 3 patients are in CR and 1 patient developed CNS relapse and underwent a second alloHSCT. Overall, 11 of 22 evaluable patients are alive in CR with full donor chimerism (50%) and an additional 2 patients are alive after second alloHSCT for CNS/extramedullary relapse.

After a median follow up of 26.7 months (range, 3.4 to 49.1) 6 patients relapsed and 9 died. All of the 6 patients who relapsed had high-risk disease (3 underwent transplantation with persistent disease, 1 had mixed-lineage leukemia gene translocation (MLL) rearrangement, 1 had FMS-like tyrosine kinase 3 (FLT3) mutation, and 1 had ETV6 gene amplification with persistent dysplasia after induction chemotherapy). Only 1 of the relapsed patients had evidence of mild acute GVHD. The 2-year OS and DFS were 56% (95% confidence interval [CI], 38% to 83%) and 48% (95% CI, 30% to 75%), respectively (Figure 2). After adjusting for competing risk of NRM, the 2-year cumulative incidence of relapse was 28% (95% CI, 8% to 48%). Causes of death included relapse (n = 4), infectious complications (n = 3), and GVHD (n = 2; 1 from acute gut GVHD, 1 from chronic lung GVHD). Among 6 patients who relapsed, 2 are alive after second alloHSCT and 4 died from their disease. Only 1 of the relapsed patients completed all 8 cycles of decitabine; others received 1 to 4 cycles. Pretransplantation conditioning chemotherapy did not appear to affect the results.

Acute and Chronic GVHD

Five patients had resolving grade I and II classic acute GVHD at the time of starting the study, and all of those completely resolved while on decitabine, with taper of steroids. One patient developed stage I gut GVHD during the second cycle of decitabine that completely resolved after a brief course of high-dose steroids with rapid taper, and another patient developed stage IV gut GVHD coinciding with the first cycle of decitabine, requiring high-dose steroids, that also completely resolved while on decitabine. One patient developed stage IV gut GVHD immediately after the first cycle, was taken off the study, and eventually died from progressive acute gut GVHD. One patient had late-onset acute gastrointestinal (GI) GVHD that resolved.

Among 9 patients who completed all cycles of decitabine, 5 have developed chronic GVHD: 2 have severe and 3 patients have moderate chronic GVHD (Table 3). Among 13 patients who discontinued decitabine earlier, 10 are at risk for chronic GVHD (2 patients died of early relapse and 1 patient died from acute gut GVHD). Seven of those 10 patients have developed chronic GVHD: 5 have severe chronic GVHD (1 with features of overlap syndrome) and 2 have moderate.

Toxicity Assessment

All 24 patients enrolled on the study were assessed for toxicity. Generally, study treatments were well tolerated (Table 4). Grade 3 and 4 hematological toxicities were experienced by 18 of 24 patients (75%), including all 6 patients treated at the 15 mg/m^2/day dose level. However, typically hematological toxicities resolved quickly between treatment cycles and accounted for very few treatment delays. The only frequently occurring grade 3 and 4 nonhematological toxicities were infections, which occurred in 8 of 24 patients (33%). Five patients discontinued the study because of the toxicities: 2 of them for infection, 1 for neutropenia, 1 for bowel obstruction, and 1 for fatigue. Among 3 patients who discontinued decitabine because of infections, only 1 was neutropenic when infection developed.

Correlative Studies

We examined the effect of decitabine on lymphocyte subpopulations, such as T cells, B cells, NK cells, and Tregs, in all the patient samples, using flow cytometry (Figures 3 and 4). Although we observed a trend toward increase of

Table 2
Summary of Outcomes

Cohort (Dose)	Patient No.	No. of Cycles	DLT	Reason for Discontinuation	Relapse	DLI	PFS, mo	OS, mo	Acute GVHD	Chronic GVHD, Grade	Cause of Death
1 (5 mg/m^2)	1	4	No	Relapse	After 4 cycles	No	5.9	28.6	No	Yes, severe	Relapse
	2	2	Yes	Relapse	After 2 cycles	1 mo after last dose	2.6	4.4	No	N/A	Relapse
	3	6	No	Noncompliance	No	No	23.0	23.0	Yes	Yes, severe	Infection
	4	1	No	Relapse	After 1 cycle	3 mo after last dose	1.5	6.8	No	N/A	Relapse
	5	8	No	Completed	No	No	49.1+	49.1+	No	No	Alive
	6	8	No	Completed	No	No	48.8+	48.8+	No	No	Alive
	7	2	N/A	Noncompliance	1 yr after second cycle	19 mo after last dose	12.3	24.7	No	Yes, severe	Relapse
	8	8	No	Completed	No	No	47.7+	47.7+	No	Yes, moderate	Alive
	9	1	No	Patient withdrew	No	No	44.9+	44.9+	No	Yes, severe	Alive
2 (7.5 mg/m^2)	10	3	No	Toxicity: infection	No	No	7.7	7.7	No	No	Infection
	11	8	No	Completed	1 yr after eighth cycle	No, second AlloHSCT	23.1+	40.4+	Yes	No	Alive
	12	2	No	PI decision	No	No	35.2+	35.2+	No	No	Alive
	13	8	No	Completed	No	No	35.5+	35.5+	No	Yes, severe	Alive
3 (10 mg/m^2)	14	8	No	Completed	No	No	34.1+	34.1+	Yes	Yes, moderate	Alive
	15	8	No	Completed	No	No	33.9+	33.9+	Yes	Yes, severe	Alive
	16	8	No	Completed	No	No	31.6+	31.6+	Yes	Yes, moderate	Alive
	17	2	No	Toxicity: ileus, infection	No	No	18.8	18.8	Yes	Yes, severe	Lung GVHD
4 (15 mg/m^2)	18	2	No	Toxicity: infection	No	No	4.7	4.7	Yes	No	Infection
	19	4	No	Toxicity: neutropenia	1 yr after fourth cycle	No, second alloHSCT	15.2+	21.2+	No	Yes, moderate	Alive
	20	6	No	Toxicity: fatigue	No	No	19.7+	19.7+	No	Yes, moderate	Alive
	21	8	N/A	Completed	No	No	13.1+	13.1+	Yes	No	Alive
	22	1	No	Acute gut GVHD	No	No	3.4	3.4	Yes	N/A	Acute gut GVHD

DLI indicates donor lymphocyte infusion; PFS, progression-free survival; N/A, not available; PI, principal investigator.

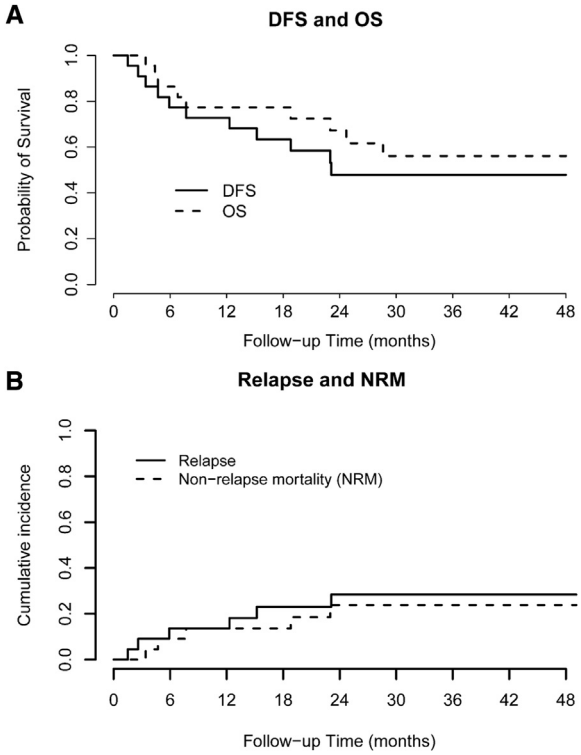


Figure 2. (A) Disease-free survival (DFS) and overall survival (OS). (B) Relapse and non-relapse mortality.

Tregs after decitabine treatment, the difference was not statistically significant. In addition, a total of 16 CpG sites of the FOXP3 locus in CD4$^+$ T cells isolated from the cohort treated with the highest dose of decitabine (15 mg/m^2/day) were analyzed, as described in Methods. The gene structure of the FOXP3 locus is presented in Supplemental Figure 1. As shown in Figure 5, we found that there is no statistically significant difference in the degree of DNA methylation in the FOXP3 locus before (C1D1 and cycle 3 day 1) and after (C1D5 and cycle 3 day 5) treatment and EOS, even though there was a trend toward decrease in DNA methylation in cycle 1. C1D5 sample of patient S101 shows a decrease in DNA methylation when compared with C1D1 sample of the same patient. Accordingly, its FOXP3 expression is higher than that of C1D1 sample (15.7% versus 11.8% FOXP3$^+$ Tregs among CD4$^+$ T cells). All of these data indicate that our DNA methylation data is consistent with the FOXP3 protein expression data. Interestingly, we found that NK cells and CD8 T cells are more sensitive to decitabine treatment in vivo with a consistent quantitative decrease in these subsets after treatment,

Table 3
GVHD Rates

GVHD Type	No. with GVHD/No. at risk
Classic acute GVHD	
Grade I-II	6/22
Grade III-IV GI	2/22
Late-onset acute GVHD	1/22
Chronic GVHD	
Moderate	
Pt completed 8 cycles	3/9
Pt completed < 8 cycles	2/10
Severe	
Patient completed 8 cycles	2/9
Patient completed < 8 cycles	5/10*

* One of these 5 patients had overlap features of GVHD.

Table 4
Grade 3 and 4 Adverse Events

Event	Grade
Anemia	3
Lymphopenia	2
Neutropenia	11
Thrombocytopenia	13
Hypotension	1
Pain, not specified	2
Diarrhea	1
SGOT (AST)	1
SGPT (ALT)	2
Infection/sepsis	8
Hypokalemia	1
Hyponatremia	1
Neuropathy	2
Dyspnea	1
Renal failure	1

SGOT indicates serum glutamic oxaloacetic transaminase; AST, aspartate aminotransferase; SGPT, serum glutamic pyruvic transaminase; ALT, alanine aminotransferase.

whereas CD4 T cells were relatively resistant to decitabine treatment (Figures 3 and 4). The CD4 T cell number at EOS in the 5 mg/m^2/day cohort is higher than that at cycle 3 day 1. The explanation for this observation is unknown and may simply represent a more rapid recovery of CD4 T cells compared to other T and NK cells after treatment.

DISCUSSION

Disease recurrence after alloHSCT is the major cause of treatment failure for patients with AML and MDS. Treatment options for those patients are limited, responses poor, and prognosis dismal. Because most of relapses occur in the first year after alloHSCT, preventive maintenance therapy should be considered early in the post-transplantation course. De Lima et al. first described that AZA maintenance after reduced-intensity alloHSCT for high-risk AML and MDS is well tolerated and may prolong event-free and OS [13].

In this study, we examined the toxicity and responses to low-dose decitabine as a maintenance therapy after alloHSCT for patients with AML and MDS. To our knowledge, this is the first report of decitabine use in this setting. We have demonstrated that decitabine can be given safely in the outpatient setting to this group of post-alloHSCT patients. Approximately 67% of our patients were able to receive at least 4 cycles of decitabine and 41% received all 8 cycles. Main DLT associated with decitabine use is myelosuppression [45]. The interval between cycles of 6 weeks rather than 4 weeks, as commonly used for treatment of AML and MDS, was chosen to facilitate count recovery. Several patients experienced neutropenia and 1 of these qualified as DLT. Interestingly, that patient relapsed 1 month later and it is possible that the neutropenia in this patient was because of the early relapse rather than a direct effect of decitabine.

All patients treated at the highest dose level (15 mg/m^2/day) experienced grade 3 or 4 hematological toxicities, none qualifying for DLT. However, 1 patient from the last cohort was discontinued after 4 cycles because of neutropenia and the other required growth factor support. Therefore, although no formal MTD was reached: 10 mg/m^2/day may be a more optimal and better tolerated dose for decitabine maintenance in post-alloHSCT setting.

Three patients were discontinued from the study because of relapse. All of them had high-risk disease and 2, in retrospect, already had early signs of relapse at the time of enrollment (manifested as mixed chimerism and evidence of multilineage dysplasia). Three additional patients, also with high-risk disease, relapsed later, after being off decitabine. Interestingly, only 1 patient who completed all 8 cycles of decitabine relapsed. Longer administration of decitabine was associated with prolonged OS and DFS.

Although decitabine could have been started between day +50 and +100 after transplantation, the majority of patients started close to day +100. The study required bone marrow biopsy within 2 weeks of starting decitabine. In some instances, treating physicians were waiting for the "standard-of-care" 3-month post-alloHSCT biopsy, rather than obtaining the "study" biopsy earlier. Many of those patients had blood counts adequate to start decitabine closer to day +50 and, in retrospect, should have been biopsied and, if eligible, enrolled.

Because most of our patients started decitabine around day +100 after alloHSCT, it is difficult to determine the effect

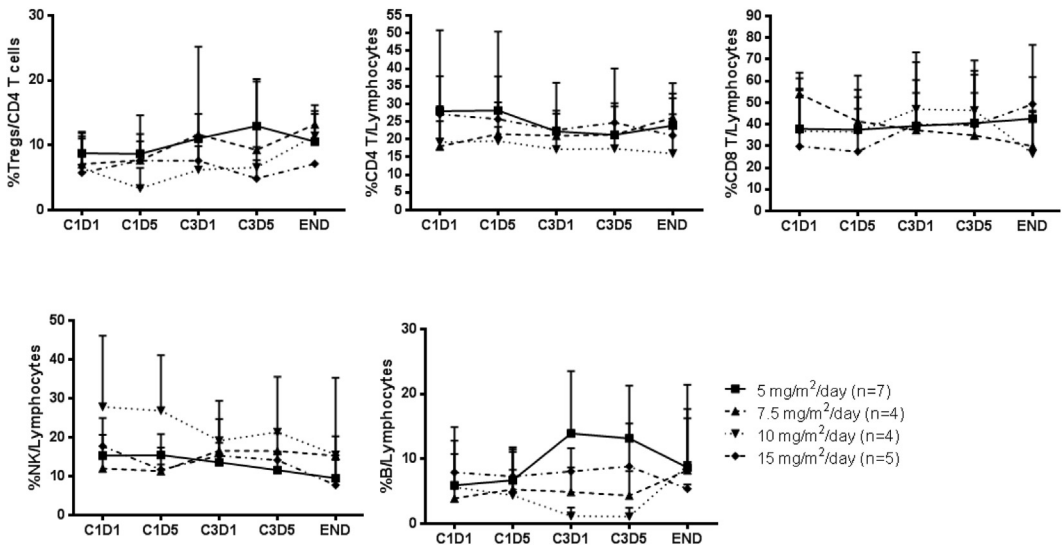


Figure 3. Effect of decitabine on the frequency of Tregs, CD4 T cells, CD8 T cells, B cells, and NK cells in the peripheral blood at stated time points (C1D1: cycle 1 day 1; C1D5: cycle 1 day 5; C3D1: cycle 3 day 1; C3D5: cycle 3 day 5; END: end of study).

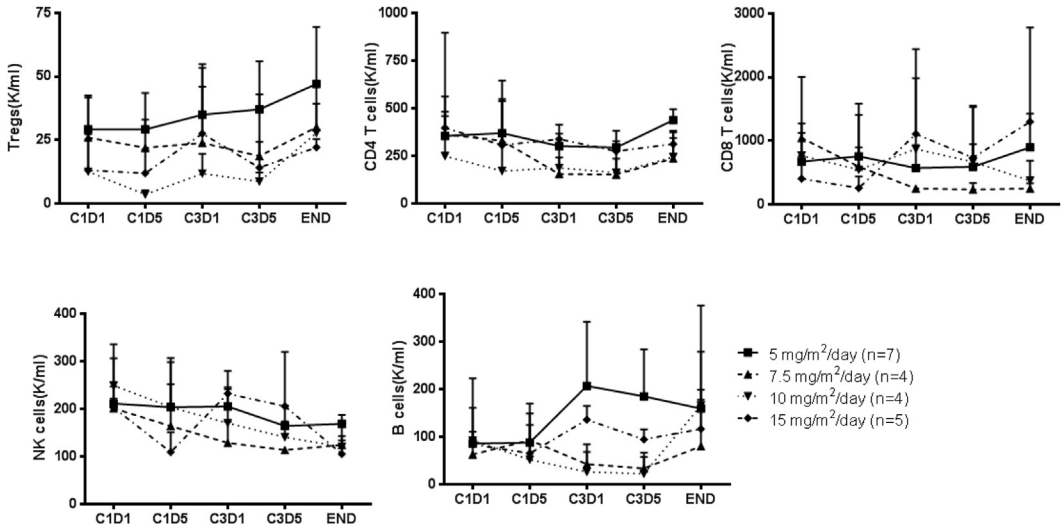


Figure 4. Effect of decitabine on the absolute number of Tregs, CD4 T cells, CD8 T cells, B cells, and NK cells in the peripheral blood and stated time points (C1D1: cycle 1 day 1; C1D5: cycle 1 day 5; C3D1: cycle 3 day 1; C3D5: cycle 3 day 5; END: end of study).

of decitabine on incidence of acute GVHD. Several patients had grade I or II acute GVHD at the time of starting decitabine, which completely resolved and 1 patient had biopsy-proven grade IV gut GVHD that started during the first week of decitabine therapy and completely resolved. However, 1 additional patient died from grade IV gut GVHD developing after the first cycle of decitabine. Decitabine maintenance did not clearly impact the rate of chronic GVHD; however, lack of comparison in this early phase study precludes any further conclusion. Although the severity of chronic GVHD appears to be lower in the group that completed all 8 cycles of decitabine, this is merely the observation; there were too few patients to suggest correlation between decitabine and chronic GVHD severity. Earlier post-transplantation initiation of decitabine might have a greater impact on the incidence of GVHD. This is another reason to support, for future studies, the use of lower decitabine dose of 10 mg/m^2 that should be better tolerated and likely result in fewer hematological toxicities and infectious complications.

Goodyear et al. used AZA maintenance after alemtuzumab-containing reduced-intensity alloHSCT, confirming the tolerability of AZA in the post-transplantation setting [35]. They observed increased number of Tregs within first 3 months after transplantation and relatively low incidence of acute and chronic GVHD. However, important differences between their study and ours are that they started AZA earlier in post-transplantation course and their patients all received alemtuzumab-containing reduced-intensity conditioning. Although we observed a trend of increased FOXP3 expression, results were not statistically significant. There are several possible explanations for why we did not observed an increase in FOXP3 expression. First, we only examined peripheral blood. Considering that most

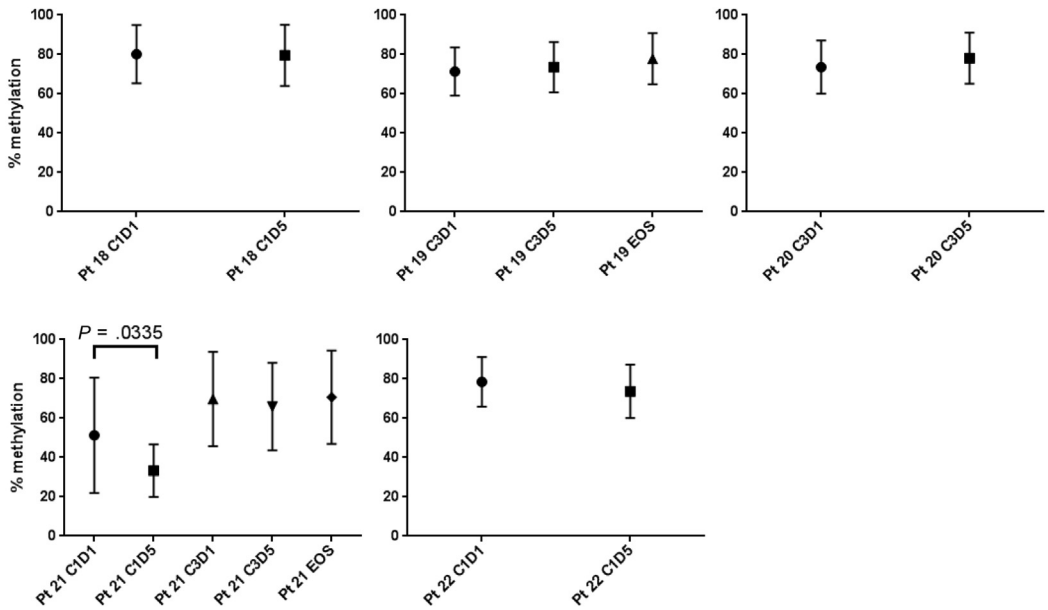


Figure 5. DNA methylation status at the FOXP3 locus of cohort 4 (15 mg/m^2/days). Shown are means and standard deviations of DNA methylation status of 16 CpGs of each patient in cohort 4 (patients 18-22). Pt indicates patient; C, cycle; D, day.

alloreactive T cells traffic to GVHD-target organs, it might be possible that those alloreactive T cells converted into FOXP3-expressing T cells might be differentially localized in the GVHD-target organs instead of the peripheral blood. Thus, it might have been more informative to examine Tregs and T cell subsets from GVHD target organs (skin, liver, and GI tract) and not the peripheral blood. Second, decitabine needs to incorporate into replicating DNA to block DNMT-1. Therefore, unless alloreactive T cells are actively proliferating, the degree of incorporation of decitabine into DNA would be limited, thereby minimizing conversion of alloreactive T cells into suppressive FOXP3 expressing Tregs. Because decitabine maintenance was not performed immediately after T cell infusion (stem cell transplantation) and was performed in the context of standard GVHD prophylaxis (calcineurin inhibitors) that also limit T cell proliferation and viability, it is possible that the optimal effect of decitabine on GVHD and FOXP3 expression was not realized in this study. Finally, it is also possible that the doses of decitabine tested in this study are not optimal to convert alloreactive T cells in vivo into Tregs, although they might be optimal for reduction of GVHD and maintenance of GVL. Decitabine does appear to reduce the numbers of NK cells and CD8 T cells while maintaining a relatively low rate of leukemia relapse, suggesting possible direct antileukemia effect of decitabine in vivo and increase of tumor-specific CD8 T cells responses.

In conclusion, low-dose decitabine maintenance is associated with acceptable toxicities when given in the post-alloHSCT setting. Although MTD was not reached, the dose of 10 mg/m^2 for 5 days every 6 weeks appears to be the optimal dose rather than 15 mg/m^2. Starting decitabine closer to the stem cell and T cell infusion, when T cells are rapidly expanding in allogeneic stem cell transplant recipients, could potentially be more efficacious in limiting GVHD. This study provides essential data for subsequent studies of decitabine maintenance. The availability of oral hypomethylating agents might make this approach of post-transplantation maintenance even more attractive.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbmt.2015.05.026.

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