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CLINICAL TRIAL REPORT

A pilot pharmacologic biomarker study of busulfan and fludarabine in hematopoietic cell transplant recipients

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Abstract
Purpose Sixteen patients diagnosed with various hematologic malignancies participated in a phase II study evaluating the addition of rabbit antithymocyte globulin (rATG, Thymoglobulin®) to the hematopoietic cell transplant (HCT) conditioning regimen of IV fludarabine monophosphate (fludarabine) and targeted intravenous (IV) busulfan (fludarabine/^{T}busulfan). Our goal was to evaluate pharmacologic biomarkers pertinent to both medications in these patients.
Methods We characterized the interpatient variability of pharmacologic biomarkers relevant to busulfan, specifically busulfan concentration at steady state, and fludarabine, specifically F-ara-A area under the curve (AUC) and fludarabine triphosphate (F-ara-ATP) intracellular accumulation and concentration in separate $CD4^+$ and $CD8^+$ T-lymphocyte populations.
Results Acute and chronic graft versus host disease (GvHD) occurred in 11 patients and one patient, respectively. Four patients died before day +100 of non-relapse causes, which met the protocol stopping guidelines. The cumulative incidence of relapse was 25% at 3 year post-HCT. Interpatient variability in the busulfan- and fludarabine-relevant pharmacologic biomarkers was 2.1- to 2.5-fold. F-ara-A AUC and accumulated F-ara-ATP in $CD8^+$ cells had the highest hazard ratio for non-relapse mortality and overall survival, respectively. However, neither achieved statistical significance.
Conclusions The low rates of GvHD, particularly in its chronic form, were encouraging, and further biomarker studies are warranted to optimize the fludarabine/^{T}busulfan/rATG conditioning regimen.

Keywords Busulfan · Fludarabine · Hematopoietic cell transplant · Biomarkers · Therapeutic drug monitoring · Pharmacokinetics

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Introduction

In the context of treating patients with hematologic malignancies, the goal of allogeneic hematopoietic cell transplantation (HCT) is to improve disease-free survival with minimal regimen-related toxicity and graft versus host disease (GvHD). The high-dose busulfan/cyclophosphamide (BU/CY) conditioning regimen has substantial regimen-related toxicity, even after targeting busulfan doses to an optimal systemic exposure [1]. There is evidence that substitution of the potent immunosuppressive agent fludarabine monophosphate (Fludara®, abbreviated as "fludarabine" here) for cyclophosphamide will lower regimen-related toxicity [2–5]. Data also suggest that the additional immunosuppression by rabbit antithymocyte globulin (rATG, Thymoglobulin®) may improve engraftment and GvHD

rates [3, 5]. Current data suggest that a fludarabine/busulfan ± rATG regimen does have lower toxicity without jeopardizing engraftment compared to BU/CY, although randomized trials are lacking [3, 5].

Due to the interest in fludarabine/busulfan conditioning, we sought to characterize the interpatient variability of pharmacologic biomarkers pertinent to both medications. Although the narrow therapeutic index of busulfan is well characterized in the context of BU/CY administration, the concentration–effect relationship of busulfan varies based on the conditioning regimen, the underlying malignancy, and the age of the HCT recipient [6]. Preliminary data indicate that increased busulfan exposure is associated with greater regimen-related toxicity in patients conditioned with fludarabine/busulfan/total body irradiation [7]. Further data are needed to establish the precise range of busulfan exposure when administered in the context of a fludarabine/busulfan regimen [8].

Fludarabine is administered intravenously as a more soluble pro-drug. After administration, fludarabine is rapidly dephosphorylated to F-ara-A (9-beta-D-arabinofuranosyl-2-fluoroadenine) by nucleotidases [9–11]. F-ara-A subsequently undergoes cellular uptake and sequential phosphorylation to its active metabolite, fludarabine triphosphate (F-ara-ATP), which inhibits ribonucleotide reductase and DNA polymerase, leading to cell death [10]. Because the active compound is the intracellularly formed F-ara-ATP metabolite, it has been hypothesized that the pharmacodynamic effect of fludarabine is more related to F-ara-ATP levels in the target cells than to plasma concentrations of the parent drug [10, 12]. It is currently not possible to directly measure intracellular F-ara-ATP exposure in T-lymphocytes from HCT patients because fludarabine depletes circulating lymphocytes. Therefore, our group recently developed ex vivo methods to estimate F-ara-ATP accumulation in T-lymphocytes obtained prior to administration of the HCT conditioning regimen [13]. These results demonstrated appreciable interpatient variability in F-ara-ATP accumulation in HCT patients, showing 10.5- and 12.5-fold variation in $CD4^+$ and $CD8^+$ cells, respectively [13].

With this pilot study, we sought to evaluate whether novel pharmacologic biomarkers can predict the sensitivity of a HCT patient to fludarabine administration. We also sought to characterize the interpatient variability of pharmacologic biomarkers relevant to busulfan, specifically busulfan concentrations at steady state (Css) and to fludarabine, specifically F-ara-A area under the plasma concentration time curve (AUC), as well as estimated F-ara-ATP accumulation and concentrations in separate $CD4^+$ and $CD8^+$ T-lymphocyte populations. We also conducted a pilot pharmacodynamic analysis to assess any correlation with non-relapse mortality (NRM) and overall survival of fludarabine/targeted (^{T}) busulfan/rATG regimen.

Materials and methods

Patient selection

From April to November 2006, 16 patients over 18 years of age were enrolled onto this phase II protocol. All participants had a malignant hematological disease unlikely to respond to conventional treatment and an HLA-identical donor. The conditioning regimen used was targeted intravenous (IV) daily busulfan, fludarabine, and rATG. Eleven patients (69%) had HLA-identical sibling donors, and five patients (31%) had HLA-identical unrelated donors. High-resolution typing was performed for unrelated donors, as described by Petersdorf et al. [14] for HLA matching at the allele level. The source of the stem cells for all patients was granulocyte colony-stimulating factor (G-CSF)- mobilized peripheral blood progenitor cells (PBPC).

Written consent was obtained using forms approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. This study was registered with ClinicalTrials.gov as trial identifier # NCT00346359.

Technique of hematopoietic cell transplantation

The conditioning regimen consisted of fludarabine on days −6 to −2, targeted daily IV busulfan on days −5 to −2, and rATG on days −3 to −1. We sought to determine the incidence and severity of acute GVHD (efficacy) and the incidence of engraftment (safety) of this busulfan/fludarabine/rATG regimen modified from regimens reported previously by us [2] and the Calgary group [15]. The fludarabine dose was identical to that in the Calgary regimen [15]. The target busulfan Css of 900 ± 100 ng/mL was chosen based on our historical experience with oral busulfan in the ^{T}BU/CY [1] and ^{T}BU/fludarabine regimens [2]. A larger percentage of patients achieved the target busulfan Css with an initial daily IV busulfan dose of 4 mg/kg [6] compared to 3.2 mg/kg used in the Calgary regimen [15].

The doses were calculated based on body weight and body surface area according to institutional guidelines. The first fludarabine dose was administered in the ambulatory clinic; subsequently, patients were admitted to the hospital to permit pharmacokinetics-based targeting of IV busulfan doses. The fludarabine dose was 50 mg/m^2/day, administered as a 30 min IV infusion, for 5 days. Fludarabine doses were not adjusted, regardless of biomarker data. Busulfan was infused over 3 h, with the busulfan infusion starting immediately after completion of the fludarabine infusion. The first busulfan dose was 4 mg/kg, using either adjusted ideal or actual body weight as previously described [16]. Subsequent busulfan doses (i.e., doses 2, 3, and 4) were targeted to achieve plasma Css of 900 ± 100 ng/mL.

Rabbit antithymocyte globulin (rATG, Thymoglobulin®; Genzyme Corp., Cambridge, MA) was administered IV at doses of 0.5 mg/kg on day −3, 2.5 mg/kg on day −2, and 3 mg/kg on day −1 based on actual body weight.

GvHD prophylaxis consisted of tacrolimus and methotrexate. Tacrolimus was started as a continuous IV infusion (0.02 mg/kg/h) beginning on day −1, and methotrexate was administered at doses of 10 mg/m^2 IV on days +1, +3, +6, and +11. Tacrolimus concentrations were monitored to achieve a steady state whole blood concentration of 5–10 ng/mL. Diagnoses of acute or chronic GvHD were made by established criteria [17, 18]. Generally, patients with clinically significant GvHD received steroid treatment and continued administration of tacrolimus as described previously [19].

Patients received similar antiemetics, antibiotics, and antifungal therapy per institutional Standard Practice Guidelines; corticosteroids were not used as antiemetics with fludarabine. All patients received phenytoin to prevent seizures during treatment with busulfan. Antimicrobials were administered for prophylaxis of *Pneumocystis jirovecii*, *Candida albicans*, and herpes zoster/simplex infections or reactivation. Patients were monitored for cytomegalovirus reactivation and received pre-emptive therapy with ganciclovir if necessary. Plasma samples also were monitored weekly for Epstein Barr Virus DNA, and treatment with rituximab was given if the titer exceeded 1000 copies/mL.

Neutrophil engraftment was defined as the first of three consecutive days with an absolute neutrophil count (ANC) $\geq 0.5 \times 10^9$/L. Platelet engraftment was defined as the first of seven consecutive days with the platelet count $\geq 20 \times 10^6$/L, without transfusion. Non-relapse mortality was defined as death in the absence of relapsed malignancy. Diagnoses of acute or chronic GvHD were made by established criteria [17, 18]. Relapse was defined by the presence of myeloblasts in the marrow (or blood) determined morphologically, by flow cytometry, or by the reappearance of cytogenetic abnormalities that had been present before HCT. Relapse assessments were conducted in all patients by examining marrow samples morphologically, by cytogenetic analysis, and by flow cytometric analysis around days +28 and +80, at 1 year post-transplant, and as clinically indicated.

Pharmacologic biomarkers for fludarabine

F-ara-A pharmacokinetic sampling and analysis

Pharmacokinetic samples were collected with the first fludarabine dose only on day −6. Pharmacokinetic sampling for F-ara-A was not conducted after subsequent doses (i.e., days −5 to −2), because these fludarabine doses were administered immediately before intensive IV busulfan pharmacokinetic monitoring of which was required for personalizing busulfan doses (see "Pharmacologic biomarkers for busulfan" below). Notably, F-ara-A clearance does not change with busulfan co-administration [20].

F-ara-A pharmacokinetic samples were drawn at 0.5, 0.583, 1.5, 4.5, 6.5, and 24 h after the start of the fludarabine infusion. The 24 h pharmacokinetic sample was drawn before the second fludarabine dose. F-ara-A plasma concentrations were quantitated using LC/MS as previously described [2]; the dynamic range was 0.23–9.04 μM, and the interday coefficient of variation was less than 10%. WinNonlin (Pharsight, Mountain View, CA) was used to calculate the AUC, clearance, and half-life ($T_{1/2}$).

Ex vivo F-ara-ATP accumulation in T-lymphocytes from patients awaiting HCT

A peripheral blood sample (60 mL) was obtained from 13 participants within the 2 weeks prior to the administration of the first fludarabine dose. This blood sample underwent a Miltenyi magnetic bead enrichment and purification procedure for $CD4^+$ and $CD8^+$ T-lymphocytes, as previously described [13]. The measurement of F-ara-ATP accumulation was initiated in each cell population independently within 2 h of cell isolation. Cells were incubated with fludarabine (5 μM) in RPMI 1640 media (not containing phenol red) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic for 4 h at 37°C in the presence of 5% CO_2. This concentration is within the range of the peak F-ara-A plasma concentration (3 μM) after fludarabine administration of 30 mg/m^2/day. Starting cell numbers varied slightly based on yield from the isolation procedure. A minimum number of 1.25×10^5 cells were needed per incubation to form adequate amounts of F-ara-ATP for accurate quantitation. Incubations were conducted in at least two replicates. After fludarabine exposure, cells were washed twice in ice-cold phosphate-buffered saline, centrifuged, solubilized, and frozen at −70°C. After thawing, the sample was centrifuged and the supernatant neutralized prior to the quantitation of F-ara-ATP using the LC–MS method described previously [13]. The units for all F-ara-ATP accumulation were pmol/1×10^6 cells/4 h.

F-ara-ATP concentrations in T-lymphocytes

The intracellular F-ara-ATP concentrations in $CD4^+$ and $CD8^+$ cells over 24 h were estimated using the ex vivo F-ara-ATP accumulation in each cell population and the plasma F-ara-A Css (defined as AUC divided by the dosing interval). Specifically, the estimated F-ara-ATP concentration within each lymphocyte population was calculated

as follows: patient's ex vivo F-ara-ATP accumulation (pmol F-ara-ATP/1 $\times$ 10^6 lymphocytes/4 h) $\times$ (lymphocyte/0.21 picoliter) [21] $\times$ (patient's F-ara-A Css/5 μM) $\times$ 24 h. This calculation of the estimated F-ara-ATP concentration is supported by data demonstrating that the intracellular accumulation rate of F-ara-ATP is linear over pharmacologically relevant fludarabine concentrations (i.e., 1.25–10 μM), lymphocyte cell counts (i.e., 1.25 $\times$ 10^5 to 2 $\times$ 10^6 cells), and incubation time (i.e., 1–5 h) [13]. Estimated F-ara-ATP concentration was not included in the statistical analysis as it is estimated from the F-ara-ATP intracellular accumulation rate and the F-ara-A AUC.

Pharmacologic biomarkers for busulfan

All involved nursing staff was trained regarding administration of IV busulfan and the subsequent flush of IV tubing to minimize dosage inconsistencies. After doses 1, 2, and 3, pharmacokinetic samples were drawn at the end of the 3-h infusion and at 3.25, 4.5, 6, 8, 11, and 24 h (i.e., prior to subsequent dose) after the start of the infusion. A total of 21 pharmacokinetic blood samples were obtained for each patient. Busulfan concentrations were analyzed by gas chromatography with mass selective detection as previously described [22]. The dynamic range was from 25 to 4,500 ng/mL, and the interday coefficient of variation was less than 8%. WinNonlin (Pharsight, Mountain View, CA) was used to calculate the AUC, clearance, half-life ($T_{1/2}$), and Css (defined as AUC divided by dosing interval) from an individual patient's concentration–time data measured through 8 h (i.e., 3, 3.25, 4.5, 6, 8 h). The target busulfan Css was 900 $\pm$ 100 ng/mL. A busulfan Css of 900 ng/mL equals an AUC of 5,260 μM min with daily administration. After calculation of the patient's clearance, the targeted subsequent doses were calculated linearly to achieve the desired Css. All busulfan dose recommendations were made within 3 h of obtaining the last pharmacokinetic sample. Successful targeting was confirmed after doses 2 and 3, with further dose adjustments as needed. The busulfan Css quantitated after doses 1, 2, and 3 were averaged to obtain an overall busulfan Css. The total busulfan dose was calculated and divided by the adjusted ideal body weight and 4 days (i.e., the duration of busulfan administration) to give the average daily busulfan dose (mg/kg).

Statistical analysis

Clinical outcomes of interest were graft rejection, NRM, acute and chronic GvHD, relapse, and overall survival. Survival curves were estimated using the Kaplan–Meier method. Cumulative incidence curves were used to estimate the probabilities of time-to-event outcomes. Analysis of risk factors for time-to-event outcomes was performed using Cox regression. Relapse of the underlying malignancy was treated as a competing risk in the analysis of NRM. Death was a competing risk for the analysis of relapse and GvHD.

The target accrual for this phase II study was 40 participants. This sample size provided 86% power to demonstrate an improvement in the incidence of acute grade II–IV GvHD relative to the historical incidence of 70% with BU/CY conditioning and cyclosporine/methotrexate GvHD prophylaxis, if the true incidence was 50%. A stopping rule for NRM provided for discontinuation of the study if there was ever 80% confidence that the true rate of NRM before day 100 exceeded 10%. The study was terminated early after 16 patients were enrolled because this stopping rule was met.

We analyzed the Spearman correlation between F-ara-A AUC with total busulfan dose (mg/kg) and three pharmacologic biomarkers: dose 1 busulfan Css, estimated ex vivo F-ara-ATP in $CD4^+$ T-lymphocytes, and estimated ex vivo F-ara-ATP in $CD8^+$ T-lymphocytes. In addition, we analyzed associations of NRM and overall survival with the plasma F-ara-A AUC, ex vivo F-ara-ATP accumulation in $CD4^+$ T-lymphocytes, F-ara-ATP accumulation in $CD8^+$ T-lymphocytes, and average busulfan Css. The pharmacodynamic analyses were conducted by dividing the patients into two groups based on the median value for each biomarker. Hazard ratios (HR) for the development of NRM and overall mortality were calculated using Cox regression models with 95% confidence intervals (CI). Statistical analyses were performed using SAS software (Cary, NC, USA).

Results

Patient population

Sixteen patients, aged 33–65 years (median 52) were enrolled. Seven of the patients were men. The median (range) time from diagnosis to transplantation was 9 (5–29) months. The risk of relapse was defined using conventional methods [23]. Seven patients (44%) had good risk diagnoses: five patients had acute myelogenous leukemia (AML) in first complete remission, one patient had chronic myelogenous leukemia in first chronic phase, and one had myelodysplastic syndrome (MDS)—refractory anemia. Nine patients (56%) had poor risk diagnoses: five had myelofibrosis (either primary or associated with a myeloproliferative disorder), one had MDS/AML, one had hypoplastic MDS, one had MDS—refractory anemia with excess blasts, and one had chronic myelomonocytic leukemia. The HCT-comorbidity index

was low (score = 0) in three patients (19%), intermediate (score = 1–2) in seven patients (44%), and high (score > 2) in six patients (38%). All donors were HLA-matched (10/10); 11 donors were related and five were unrelated.

Clinical outcomes

All patients achieved neutrophil engraftment at a median of 16 (range 11–28) days after HCT. Fourteen patients achieved platelet engraftment at a median of 13 (range 11–13) days. Two patients remained platelet transfusion-dependent until their deaths from non-relapse causes at days +36 and +76.

One patient who was transplanted from an unrelated donor developed hyperacute GvHD of the skin on day +9 that responded completely with a short course of corticosteroid treatment. Ten of the remaining 15 patients developed mild grade IIa [24] GvHD (Fig. 1a) of the upper gastrointestinal tract at a median of 38 (range 21–98) days post-transplant. Seven patients were treated effectively with a short course of prednisone (1 mg/kg/day) plus oral non-absorbable steroids (beclomethasone, budesonide, or both), and three patients were treated with non-absorbable steroids alone [25]. None of the patients developed grade III or IV GvHD. One patient developed extensive chronic GvHD at day +175.

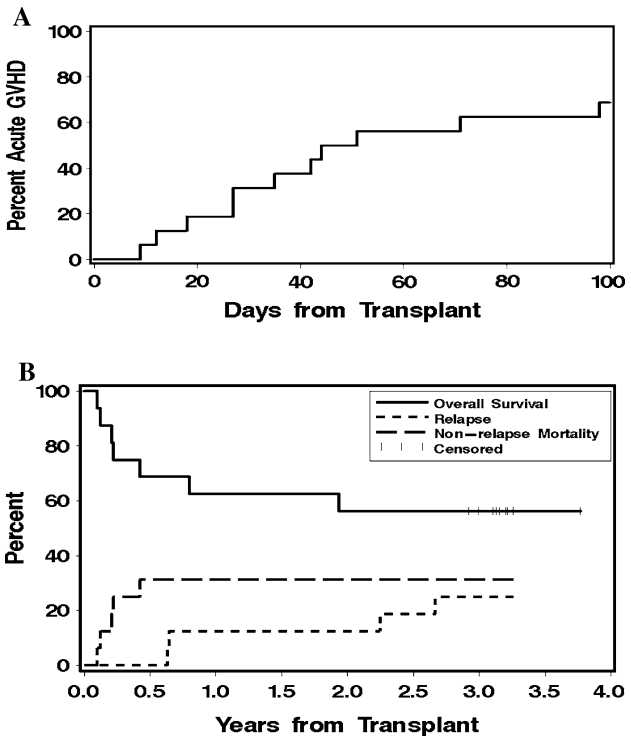

Fig. 1 Clinical outcomes of overall population. Specifically acute GvHD (**a**) and overall survival, non-relapse mortality, and relapse (**b**)

Four of 16 patients died before day +100 of non-relapse causes, which met the protocol stopping guideline for NRM; therefore, the study was closed to further accrual. Causes of death in these four patients were infection (*Pseudomonas* pneumonia), suspected infection with septic shock and disseminated intravascular coagulation, pancreatitis with multi-organ failure, and idiopathic pneumonia syndrome associated with diffuse alveolar hemorrhage.

Median follow-up for survivors was 3.1 years. As shown in Fig. 1b, the cumulative incidence of relapse was 25% (95% CI 4–46%) at 3 year post-transplant. The Kaplan–Maier estimate of overall survival was 56% (95% CI 32–81%) at 3 years.

Pharmacologic biomarkers

The pharmacologic biomarkers for fludarabine each showed considerable interpatient variability, as listed in Table 1. There was a 1.9- and 2.0-fold range in the variability of F-ara-ATP intracellular accumulation in $CD4^+$ and $CD8^+$ cells, respectively, with a 2.5-fold range in F-ara-A AUC.

Busulfan Css after dose 1, which was based on body weight, varied 2.2-fold (Table 1). The target busulfan Css of 900 ± 100 ng/mL was achieved in all patients, and there was lower interpatient variability in overall busulfan Css.

F-ara-A AUC was correlated with the busulfan Css after dose 1, which was not adjusted based on the patient's busulfan clearance. As subsequent busulfan doses were personalized, we did not evaluate the correlation between busulfan Css after dose 2 and 3. We evaluated the correlation between the daily busulfan dose (mg/kg) averaged over the 4 days of busulfan administration. F-ara-A AUC was modestly correlated with both busulfan Css after dose 1 ($R^2 = 0.50$, $P = 0.002$, Fig. 2a) and average daily busulfan dose ($R^2 = 0.34$, $P = 0.02$, Fig. 2b). F-ara-A AUC was not correlated with accumulation of F-ara-ATP in $CD4^+$ cells (Fig. 3a, $R^2 = 0.02$, $P = 0.63$) or $CD8^+$ cells (Fig. 3b, $R^2 = 0.14$, $P = 0.20$).

Pharmacodynamic relationships

We sought to evaluate wether NRM or overall survival could be predicted by the pharmacologic biomarkers of busulfan and fludarabine. Values of the biomarkers above the median were associated with higher but not statistically significant rates of NRM for F-ara-ATP accumulation in $CD4^+$ cells (HR = 3.9, 95% CI 0.4–38, $P = 0.20$), F-ara-ATP accumulation in $CD8^+$ cells (HR = 2.5, 95% CI 0.3–25, $P = 0.39$), F-ara-A AUC (HR = 5.2, 95% CI 0.6–46, $P = 0.10$), and busulfan Css (HR = 2.4, 95% CI 0.4–15, $P = 0.32$). Values above the median were also

Table 1 Description of pharmacologic biomarkers and clinical outcomes

Patient #	F-ara-A AUC (μM × h)	F-ara-ATP intracellular accumulation (pmol/ 1×10^6 cells/4 h)		F-ara-ATP intracellular concentration[a] (μM)		Busulfan Css (ng/mL)		Average daily busulfan dose (mg/kg)	Cause of death (months)	Duration of follow-up (months)
		$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	Dose 1	Overall			
1	20.4	11.6	11.5	0.566	0.559	922	956	3.88		45
2	34.1	N/A[b]	N/A	N/A	N/A	965	958	3.99	Relapse (23.2)	
3	30.2	N/A	N/A	N/A	N/A	870	923	4.00		36
4	22.8	14.3	12.1	0.774	0.654	896	934	3.85		37
5	39.9	N/A	N/A	N/A	N/A	1,548	933	2.53	Pancreatitis with multi-organ failure (2.5)	
6	25.2	12.7	17.6	0.761	1.058	798	953	4.28	Suspected infection with septic shock and DIC[c] (1.5)	
7	25.0	13.4	15.0	0.796	0.892	743	906	4.67	Invasive Zygomycosis (5.1)	
8	24.6	14.2	17.8	0.835	1.045	1,031	933	3.89		39
9	23.5	14.8	12.7	0.828	0.710	796	936	4.33	Pseudomonas pneumonia (2.6)	
10	24.1	13.2	13.9	0.756	0.799	825	844	4.00	Relapse (N/A)	
11	16.3	12.9	15.8	0.501	0.610	704	904	4.31		38
12	22.0	14.3	12.8	0.745	0.670	857	828	4.16		38
13	20.5	11.6	10.5	0.567	0.513	791	908	4.07		38
14	25.0	18.7	11.2	1.112	0.666	896	957	3.22	IPS with DAH[d] (1.2)	
15	26.5	9.7	10.2	0.609	0.644	1,026	913	3.66		37
16	37.4	11.4	8.7	1.011	0.772	943	934	3.71		39
Median (range)	24.8 (16.3–39.9)	13.2 (9.7–18.7)	12.7 (8.7–17.8)	0.761 (0.501–1.11)	0.670 (0.513–1.06)	883 (704–1,548)	933 (828–958)	4.00 (2.53–4.67)		
Fold range	2.5	1.9	2.0	2.2	2.1	2.2	1.2	1.8		

[a] F-ara-ATP intracellular concentration calculation described in "Materials and methods"

[b] N/A = not available

[c] Disseminated intravascular coagulation

[d] Idiopathic pneumonia syndrome associated with diffuse alveolar hemorrhage

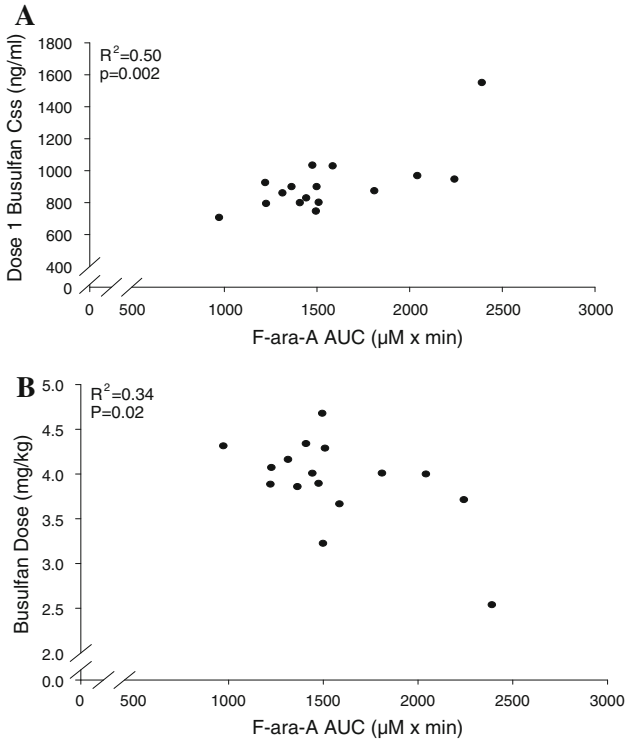

Fig. 2 Association of F-ara-A AUC with dose 1 busulfan Css (**a**) and average daily busulfan dose (**b**)

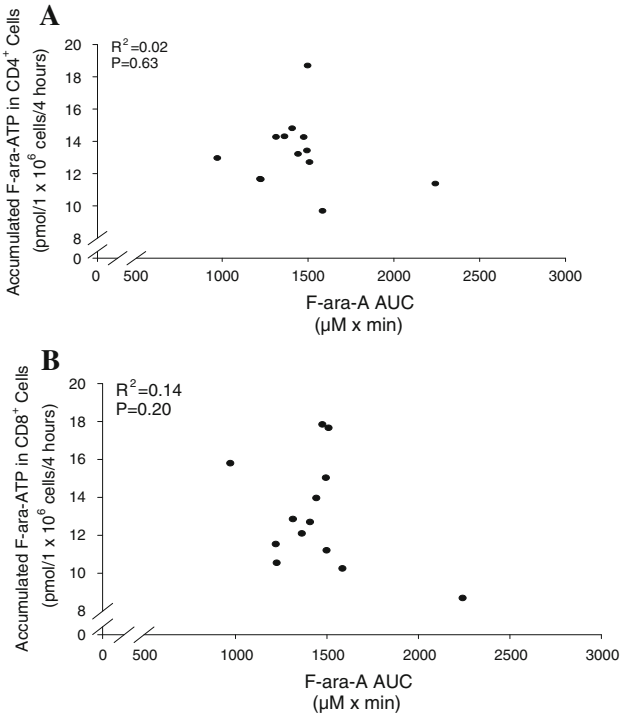

Fig. 3 Association of F-ara-A AUC with accumulated F-ara-ATP in $CD4^+$ cells (**a**) and $CD8^+$ cells (**b**)

associated with higher but not statistically significant rates of overall mortality for F-ara-ATP accumulation in $CD4^+$ cells (HR = 2.1, 95% CI 0.4–13, $P = 0.40$), F-ara-ATP accumulation in $CD8^+$ cells (HR = 3.7, 95% CI 0.6–49, $P = 0.19$), F-ara-A AUC (HR = 3.4, 95% CI 0.7–18, $P = 0.12$), and busulfan Css (HR = 2.1, 95% CI 0.5–9.7, $P = 0.31$). The biomarkers with the highest (F-ara-A AUC) and lowest hazard ratios (F-ara-ATP accumulation in $CD8^+$ cells) with NRM are shown as Fig. 4a and b, respectively. Regarding overall survival, the biomarkers with the highest (F-ara-ATP accumulation in $CD8^+$ cells) and lowest hazard ratios (busulfan Css) are shown as Fig. 5a and b, respectively.

Discussion

The incidence of severe acute GvHD and extensive chronic GvHD with this fludarabine/targeted busulfan/rATG regimen was low and consistent with results of Russell et al. [3] using a similar conditioning regimen. The majority of the acute GvHD was mild and restricted to the upper gastrointestinal tract, and the peak severity was mild (grade IIa) [24]. None of the patients experienced grade III or IV acute GvHD. Notably, the rate of chronic GvHD was 6% (i.e., one of 16), which is considerably lower than the 49% incidence in a previous trial of fludarabine/^{T}busulfan without rATG [2]. Four of 16 patients died of non-relapse causes before day +100. The addition of rATG, the increased immunosuppressive doses of fludarabine, and/or the high HCT-comorbidity index score of 3 in two of the four patients who died could have been contributory. Although the rate of relapse in patients conditioned with fludarabine/^{T}busulfan/rATG was low, overall and disease-free survivals were lowered by the unexpectedly high rate of NRM in this study compared to other studies [3, 5].

We sought to identify biomarkers associated with NRM or overall survival in this phase II study. Thus, we characterized the interpatient variability and pharmacodynamics of four separate pharmacologic biomarkers: busulfan Css, F-ara-A AUC, and ex vivo F-ara-ATP accumulation in $CD4^+$ and $CD8^+$ T-lymphocyte populations in HCT recipients conditioned with fludarabine/^{T}busulfan/rATG. None of these biomarkers were associated with NRM or overall survival (Figs. 4, 5); however these results should be considered preliminary in view of the small sample size and the heterogeneity of the patient population. The majority of pharmacodynamic studies with fludarabine/busulfan HCT conditioning regimen have focused upon busulfan. Many HCT centers target busulfan doses based on pharmacokinetics [6]. The pharmacodynamics of busulfan differ based on the recipient age, underlying disease, and conditioning regimen [6]. Busulfan has minimal effects upon lymphocytes [26, 27]. In patients

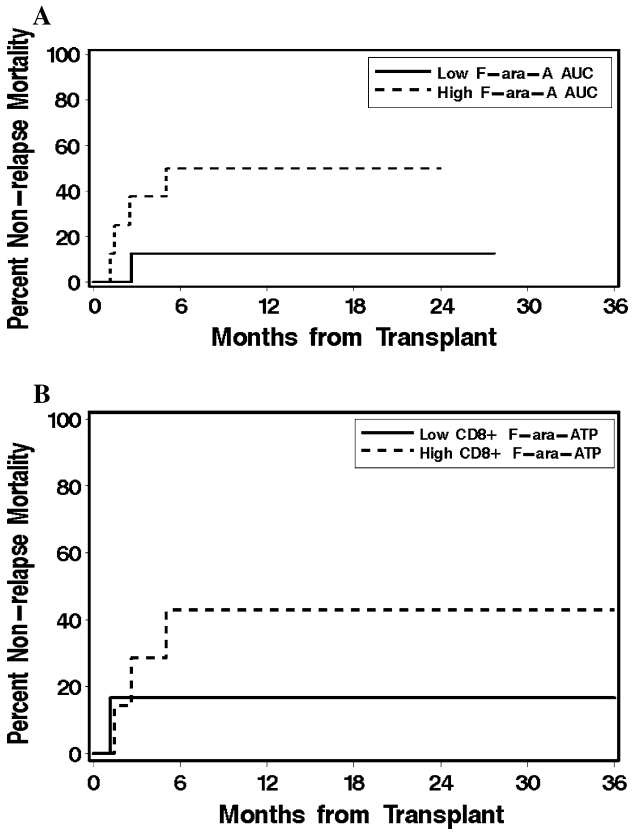

Fig. 4 Association of pharmacologic biomarkers with non-relapse mortality. **a** The biomarker with the strongest association (F-ara-A AUC; HR = 5.2, $P = 0.10$); **b** the biomarker with the weakest association (F-ara-ATP accumulation in $CD8^+$ cells; HR = 2.5, $P = 0.39$)

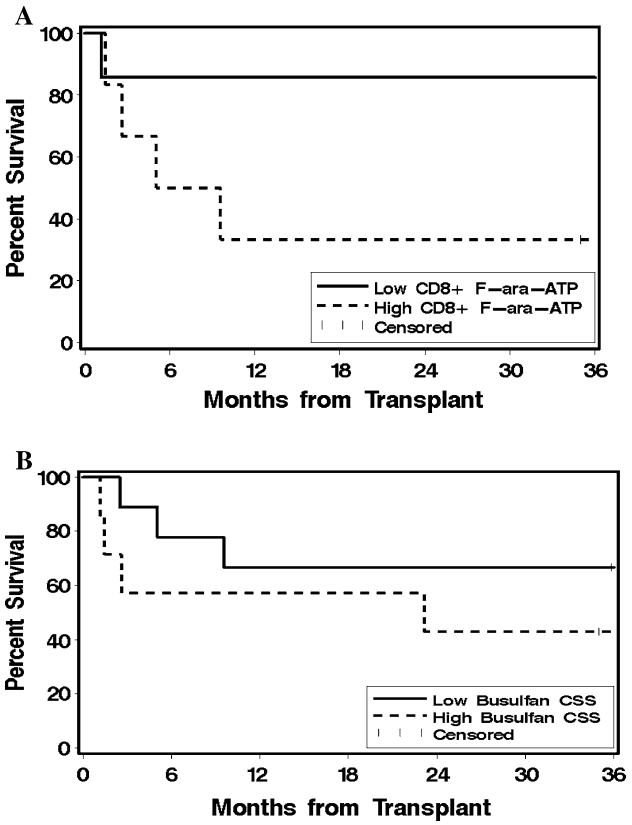

Fig. 5 Association of pharmacologic biomarkers with overall survival. **a** The biomarker with the strongest association (F-ara-ATP accumulation in $CD8^+$ cells; F-ara-AUC; HR = 3.7, $P = 0.19$); **b** the biomarker with the weakest association (busulfan Css, HR = 2.1, $P = 0.31$)

conditioned with fludarabine/busulfan/total body irradiation, Geddes et al. observed that increased busulfan exposure (daily AUC of 6,000 μM × h, which equates to a Css of 855 ng/mL) is associated with worse overall survival and increased toxicity [7]. A busulfan Css of 900 ± 100 ng/mL was targeted in our population receiving fludarabine/^{T}busulfan/rATG, demonstrating the ability to personalize daily IV busulfan doses from concentration–time data obtained over 8 h from the start of the infusion [28]. Fourteen of the 16 patients had a busulfan Css above the threshold Css of 855 ng/mL, which was associated with increased regimen-related toxicity by Geddes et al. [7]. Busulfan Css did not, however, appear to be associated with NRM or overall survival (Fig. 5b), potentially because of the narrow range of busulfan Css values. Alternatively, the pharmacodynamics may differ between the fludarabine/busulfan/total body irradiation regimen evaluated by Geddes et al. and the fludarabine/^{T}busulfan/rATG regimen evaluated in this manuscript. The sample size was limited, and additional data will be needed to further define the pharmacodynamics of busulfan when administered in combination with fludarabine. In addition, our patient population was heterogeneous in regards to underlying disease and disease status at transplantation, each of which could impact HCT outcomes. With the increasing use of this regimen, biomarker studies should not focus on busulfan alone but should also include fludarabine.

We also sought to identify pharmacology biomarkers relevant to the nucleoside analog fludarabine. F-ara-A AUC showed a 2.5-fold variability, suggesting that the interpatient variability in F-ara-A clearance is comparable to that of busulfan when busulfan is dosed based on body weight [6]. The modest correlation between F-ara-A AUC and busulfan Css (Fig. 2a) is surprising as F-ara-A is predominantly renally eliminated while busulfan primarily undergoes glutathione conjugation. Further pharmacodynamic studies are needed to determine whether F-ara-A AUC is associated with clinical outcomes or whether targeting doses of fludarabine would provide clinical benefit. We have taken that approach with busulfan [29] and cyclophosphamide [30, 31]. With both of these alkylating agents, the pharmacodynamics differ based on the conditioning regimens. Preliminary data suggest similar conditioning regimen-dependent pharmacodynamic relationships

with F-ara-A. For example, F-ara-A AUC was not related to T cell chimerism or engraftment in patients receiving fludarabine/^{T}busulfan conditioning [2]. In patients conditioned with fludarabine/cyclophosphamide/total body irradiation, those with low F-ara-A AUCs were less likely to experience neutrophil engraftment, while the one patient with the highest F-ara-A AUC died due to neurotoxicity [32]. Further studies testing the hypothesis that interpatient variability of clinical outcomes is related to F-ara-A AUC should be conducted in a homogenous group of patients conditioned with fludarabine/^{T}busulfan/rATG. The development of a population pharmacokinetic model and limited sampling schedule to characterize F-ara-A AUC with the fewest number of pharmacokinetic samples may facilitate such studies [33]. Our results show that F-ara-A AUC is weakly correlated with accumulation of F-ara-ATP in $CD4^+$ and $CD8^+$ cells (Fig. 3a, b); thus, measurement of all three pharmacologic biomarkers is necessary.

In conjunction with characterizing F-ara-A AUC, we sought also to evaluate the role of intracellular F-ara-ATP in non-relapse and overall mortality. Subsequent to administration, fludarabine is quickly dephosphorylated to F-ara-A by nucleotidases [9–11]. F-ara-A then undergoes cellular uptake and sequential phosphorylation to its monophosphate, diphosphate, and triphosphate metabolites [10]. The inhibition of ribonucleotide reductase and DNA polymerase by F-ara-ATP, the active metabolite, ultimately leads to cellular apoptosis [10]. We have recently developed a novel ex vivo method to phenotype F-ara-ATP accumulation in $CD4^+$ and $CD8^+$ T-lymphocytes isolated from individual patients [13]. Intracellular F-ara-ATP accumulation, in conjunction with F-ara-A AUC, can be used to estimate F-ara-ATP concentrations in $CD4^+$ and $CD8^+$ cells (Table 1). We hypothesize that greater intracellular F-ara-ATP accumulation in $CD8^+$ lymphocytes leads to greater lymphosuppression, with downstream effects of higher infection rates, higher non-relapse mortality, and inferior overall survival (Fig. 5a). Our preliminary results suggest that F-ara-ATP accumulation in $CD8^+$ cells may predict overall survival, although sample size is limited and further data are needed.

In conclusion, there is considerable interpatient variability in the pharmacologic biomarkers relevant to fludarabine. However, no pharmacodynamic association was clear based on this pilot study in patients. Further studies evaluating the association of these biomarkers are needed in a homogeneous population of patients receiving fludarabine/^{T}busulfan/rATG conditioning.

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