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Cyclophosphamide followed by intravenous targeted busulfan for allogeneic hematopoietic cell transplantation: pharmacokinetics and clinical outcomes

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Abstract

Targeted busulfan/cyclophosphamide (^{T}BU/CY) for allogeneic hematopoietic cell transplantation (HCT) carries a high risk of sinusoidal obstruction syndrome (SOS) in patients transplanted for myelofibrosis. We tested the hypothesis that reversing the sequence of administration (from ^{T}BU/CY to CY/^{T}BU) will reduce SOS and day +100 non-relapse mortality (NRM). We enrolled 51 patients with myelofibrosis (n=20), acute myeloid leukemia (AML, n=20), or myelodysplastic syndrome (MDS, n=11) in a prospective trial of CY/^{T}BU conditioning for HCT. Cyclophosphamide 60 mg/kg/day IV for two days was followed by daily IV BU for four days, targeted to a concentration at steady state (Css) of 800–900 ng/mL. CY/^{T}BU-conditioned patients had higher exposure to CY ($p<0.0001$) and lower exposure to 4-hydroxyCY ($p<0.0001$) compared to ^{T}BU/CY-conditioned patients. Clinical outcomes were compared with controls (n=271) conditioned with ^{T}BU/CY for the same indications. In patients with myelofibrosis, CY/^{T}BU conditioning was associated with a significantly reduced incidence of SOS (0% vs. 30% after ^{T}BU/CY, $p=0.006$), while SOS incidence was low in both cohorts with AML/MDS. Day +100 mortality was significantly lower in the CY/^{T}BU cohort (2% vs. 13%, $p=0.01$). CY/^{T}BU conditioning markedly impacted CY pharmacokinetics and was associated with significantly lower incidences of SOS and day +100 mortality, suggesting that CY/^{T}BU is superior to ^{T}BU/CY as conditioning for patients with myelofibrosis.

INTRODUCTION

Busulfan followed by cyclophosphamide (BU/CY) is a commonly used high-dose conditioning regimen in allogeneic hematopoietic cell transplantation (HCT). Regimen-

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related toxicity, graft rejection, and relapse in patients conditioned with BU/CY have been reduced by individualized dosing of BU to a target steady-state concentration (targeted BU/CY, ^{T}BU/CY) [1,2]. However, neither BU dose-targeting nor the introduction of intravenous ^{T}BU has eliminated hepatic sinusoidal obstruction syndrome (SOS) as a cause of morbidity and mortality [3,4]. BU is not inherently toxic to hepatocytes or to sinusoidal endothelial cells, whereas metabolites of CY, generated within hepatocytes and transported into hepatic sinusoids, are highly toxic to sinusoidal endothelial cells [5-7]. It follows that CY metabolites are the prime cause of regimen-related liver toxicity following the ^{T}BU/CY regimen.

There are several possible approaches to minimizing regimen-related toxicity caused by the combination of ^{T}BU and CY. One approach is to eliminate CY altogether, for example by using a regimen of fludarabine and BU (FLU/^{T}BU) [8,9]. A second approach is to eliminate variability in CY exposure with pharmacokinetic targeting of CY doses, which is feasible and effective in reducing toxicity [10]. A third, simpler approach is to reverse the order of administration, giving CY first, followed by IV ^{T}BU (CY/^{T}BU). The pharmacologic rationale for a CY/ ^{T}BU regimen rests on the following observations: 1) BU depletes hepatic glutathione (GSH), and at high concentrations induces oxidative stress in murine hepatocytes *in vitro* [6]; 2) glutathione is important in both the detoxification of the CY metabolite 4-hydroxycyclophosphamide (HCY) through conversion to glutathionyl-CY and in the elimination of the toxic CY metabolite acrolein [5,11]; 3) restoration of hepatic and sinusoidal endothelial cell GSH levels prevents injury to hepatic sinusoids in several different animal models of toxic liver injury [12]; and 4) studies in patients receiving high-dose conditioning regimens have suggested a reduced risk of hepatotoxicity when BU was given after, rather than before, other conditioning agents [13-15]. Thus, giving BU first appears to potentiate CY toxicity, providing the basis for administering these drugs in reverse order (CY/ ^{T}BU) to reduce toxicity.

Here, we report the results of a prospective clinical trial designed to test the hypothesis that reversed-sequence (CY/^{T}BU) conditioning reduces the frequency and severity of hepatotoxicity, compared to the standard sequence of BU followed by CY (^{T}BU/CY). Additionally, we collected pharmacokinetic data to test whether altering the sequence of conditioning agents led to measurable changes in CY metabolism and exposure to CY metabolites. We enrolled two cohorts of patients, one at high risk for toxic sinusoidal liver injury (patients with myelofibrosis) [16] and one at standard risk (patients with myelodysplastic syndrome [MDS] or acute myeloid leukemia [AML]). We compared liver toxicity and outcomes with those in concurrent and historical control patients who received ^{T}BU/CY and allogeneic HCT for the same disease indications. The primary outcome was the incidence of moderate/severe SOS after allogeneic HCT.

MATERIALS AND METHODS

Patient selection

Study patients (cases) were enrolled from 1 March 2007 through 30 June 2010 on Fred Hutchinson Cancer Research Center (FHCRC) Protocol 2130. This protocol was approved by the FHCRC Institutional Review Board (IRB) and registered at www.clinicaltrials.gov as NCT00445744. All patients provided written informed consent using forms approved by the IRB. Under the aegis of IRB-approved Protocol 881, a cohort of historical patients (controls) was obtained by retrieving clinical data on consecutive patients with myelofibrosis, AML, or MDS undergoing allogeneic HCT after ^{T}BU/CY conditioning between 1 January 2003 and 31 December 2009.

Eligibility criteria

Eligible were patients: a) with primary myelofibrosis (PMF), myelofibrosis secondary to polycythemia vera (PV) or essential thrombocythemia (ET), AML, or MDS; b) aged <61 years if transplanted from unrelated donors, or aged <66 years if transplanted from related donors; c) receiving unmanipulated G-CSF-mobilized peripheral blood mononuclear cell (G-PBMC) or G-CSF-stimulated bone marrow allograft products; d) with a Karnofsky performance status of >70% at the time of HCT; and e) able to provide informed consent. Patients were required to have an HLA-identical related donor or an HLA-matched or 1-HLA-allele-mismatched unrelated donor identified before enrollment.

Exclusion criteria included: a) HIV infection or active viral hepatitis; b) use of medications known to strongly inhibit the cytochrome P450 pathway and which, in the judgment of the attending physician, could not be safely discontinued during conditioning; c) known hypersensitivity to BU or CY; d) hepatic dysfunction as evinced by total serum bilirubin or aspartate aminotransferase >2x the upper limit of normal, or evidence of synthetic dysfunction or cirrhosis; e) renal insufficiency as evinced by creatinine clearance <50% of expected, serum creatinine >2x the upper limit of normal, or dialysis dependence; f) impaired pulmonary function as evidenced by PaO2 <70 mm Hg and DLCO <70% predicted or by PaO2 <80 mm Hg and DLCO <60%, or requirement for continuous supplementary oxygen; and g) impaired cardiac function as evinced by ejection fraction <35% or presence of symptomatic coronary artery disease.

Conditioning regimen

The conditioning regimens for protocol cases and control patients are summarized in Table 1. All patients were conditioned with CY 60 mg/kg/day IV for two consecutive days (total dose, 120 mg/kg) and targeted BU, given for four consecutive days. On the days of CY infusion, patients received MESNA (2-mercaptoethane sulfonate) at milligram doses equal to those of CY as prophylaxis against uroepithelial damage.

Cases (n=51) received CY followed by targeted IV BU (CY/^{T}BU). CY was administered IV at 60 mg/kg/day on days -7 and -6 before HCT. Targeted BU was administered intravenously as Busulfex (Otsuka; Tokyo, Japan) once daily on days -5 through -2, for a total of four daily doses. Prophylactic phenytoin was initiated on day -6 after completion of the second CY dose, and discontinued on day -1; one patient received prophylactic levetiracetam.

Patients in the control cohort (n=271) received targeted BU followed by CY (^{T}BU/CY). In this cohort, BU was administered on days -7 through -4 orally at an initial dose of 1 mg/kg every 6 hours in 252 patients (93%), intravenously at a starting dose of 0.8 mg/kg every six hours in 15 patients (6%), and intravenously at a starting dose of 3.2 mg/kg daily in 4 patients (1%). After the initial weight-based dose of BU, subsequent doses were adjusted to achieve the target plasma steady-state concentrations (Css) described in Table 1. CY was administered at 60 mg/kg/day IV on days -3 and -2. Prophylactic phenytoin was given from day -8 through day -3.

Cyclophosphamide dosing and pharmacokinetics

CY was infused through a central venous catheter. The CY dose was based on adjusted ideal body weight (0.25 × [actual weight – ideal weight] + ideal weight) if actual body weight was greater than ideal body weight [17]. The infusion duration followed FHCRC Standard Practice Guidelines: total CY doses of <5,000 mg were infused over 1 hour, and CY doses ≥ 5,000 mg were infused over 2 hours. CY doses were not adjusted based on pharmacokinetic data.

In cases (CY/^{T}BU) only, blood samples were drawn after each dose of CY from the central venous lines at the end of infusion, and at 2, 4, 8, 16, 20, and 24 hours after the start of the CY infusion. If the CY infusion lasted 1.5 hours or longer, blood samples were instead drawn at the end of infusion and at 3, 5, 8, 16, 20, and 24 hours after the start of the infusion. At each of these time points, blood was aliquoted into two tubes: one containing EDTA for analysis of CY and carboxyethylphosphoramide mustard (CEPM), and the other containing phenylhydrazine HCl to stabilize HCY, as previously described [18]. Samples were refrigerated at the bedside at a target temperature of 4° C unti l transport (within 12 hours) to the Pharmacokinetics Laboratory. Plasma concentrations of CY, HCY, and CEPM were quantified by liquid chromatography and mass spectroscopy methods [10]. Patient exposure to CY and its metabolites was calculated by determining the AUC_{CY}, AUC_{HCY}, and AUC_{CEPM} for the interval 0 to 48 hours using non-compartmental analysis. These AUCs were compared to those previously reported in patients receiving ^{T}BU/CY [18]. CY pharmacokinetics were not evaluated in the historical control patients.

Busulfan dosing and pharmacokinetics

In the 51 case patients (CY/^{T}BU), daily IV BU doses were standardized regarding the time of administration, duration of BU infusion, and administration of saline flushes within the IV line for consistent BU pharmacokinetics. In these patients, the first BU dose (day -5) was 4 mg/kg, with body weight calculated as described above [17]. All subsequent BU doses were adjusted to achieve a Css of 800–900 ng/mL.

In the 271 control patients (^{T}BU/CY), the BU administration route and target Css were chosen by the attending physician. The majority of patients received oral BU every six hours (n=252); a minority received IV BU every six hours (n=15) or as a combined single daily dose (n=4). The target Css for most patients (n=262) was 800–900 ng/mL; five patients had target Css ≤900 ng/mL and four patients had target Css >900 ng/mL.

In both cases and controls, blood samples for BU pharmacokinetics (3 mL/sample) were collected in sodium-heparin-containing tubes at the time points previously described [8]. Samples were stored on wet ice or refrigerated until transport to the laboratory, where plasma BU concentrations were analyzed by gas chromatography with mass selective detection as previously described [19]. The dynamic range was from 62 to 4,500 ng/mL and the intraday and interday coefficients of variations were <5% and <8%, respectively.

Individual patient concentration-time data were fit using WinNonlin (version 5.2). The AUC from time 0 to infinity ($AUC_{0\ to\ \infty}$) was calculated after each dose. Clearance and Css were calculated based on the following equations: clearance = dose divided by AUC, and Css = $AUC_{0\ to\ \infty}$ multiplied by BU molecular weight (246.3 g/mol) divided by the dosing interval. After calculation of each patient's clearance, subsequent dose levels were calculated linearly to achieve the target Css, as described previously [17].

Supportive care and prophylaxis

Graft-vs.-host disease (GVHD) prophylaxis consisted of tacrolimus and methotrexate. Tacrolimus was given as a continuous IV infusion beginning on day -1 at an initial dose of 0.03 mg/kg/day, with doses adjusted to achieve trough tacrolimus concentrations at steady-state of 5–15 ng/mL. Tacrolimus was converted from IV infusion to divided oral dosing as soon as tolerated. In the absence of GVHD, tacrolimus was tapered in 20% decrements starting on day +56 after HCT, to be discontinued completely by day +200. In patients with GVHD, tacrolimus was maintained at therapeutic trough concentrations with subsequent tapering and management dictated by the attending transplant physician on the basis of

clinical GVHD activity. Methotrexate was given at doses of 10 mg/m^2 IV on day +1 (at least 24 hours after donor cell infusion) and on days +3, +6, and +11.

All patients received antifungal, antiviral, and antibacterial prophylaxis per FHCRC standard practice. Hematopoietic growth factors were given only in the event of prolonged neutropenia after day +21. Ursodiol was administered orally to both cases and historical control patients at 12 mg/kg/day, starting two weeks before the initiation of conditioning, per FHCRC standard practice.

Evaluation of outcomes

All case and control patients were evaluated by two investigators (G.B.M. and A.K.) for evidence of SOS after HCT. The diagnosis of SOS was based on the occurrence (by day +20 after HCT) of at least two of the following: hyperbilirubinemia (serum bilirubin > 2.0 mg/dL); hepatomegaly or right upper quadrant pain of liver origin; or weight gain (>2% of dry body weight) due to fluid accumulation [20]. If other possible causes of liver dysfunction were present (e.g., GVHD, sepsis syndrome, drug-induced liver injury), patients were classified as having liver disease of uncertain etiology (LDUE). The severity of SOS was graded as mild (resolving without specific treatment), moderate (requiring diuretics, sodium restriction, or analgesics, but with eventual resolution of abnormalities), or severe (death or non-resolution by day +100).

Overall survival was estimated by the Kaplan-Meier method. The cumulative incidences of non-relapse mortality (NRM) and relapse were estimated by standard methods, treating these outcomes as mutually competing events. Statistical comparisons of survival, NRM, and relapse between groups used Cox regression, restricting the analysis to events within the first 100 days or first two years after HCT, as indicated. The associations of the AUC of CY and its metabolites with these outcomes were evaluated as a test for trend over quartiles using Cox regression. Statistical comparisons of the frequency of SOS were done by the chi-squared test. Comparisons of pharmacokinetic parameters between regimens were carried out using the Wilcoxon rank-sum test. Comparisons of relapse rates were adjusted using the disease-risk criteria described by Kahl *et al* [21]. Outcomes in patients with AML/MDS were compared to those in patients with myelofibrosis as part of a pre-specified subset analysis.

RESULTS

Patient demographics

Patient and disease characteristics are summarized in Table 2. The median age of cases was 55 (range, 30–65) years. Twenty patients (39%) had myelofibrosis, 11 (22%) had MDS, and 20 (39%) had AML. Two cases had undergone previous allogeneic HCT: one patient with myelofibrosis had rejected an allograft after ^{T}BU/CY conditioning 10 years earlier, and a second patient with AML had relapsed after HCT following reduced-intensity conditioning performed three months before study enrollment. The median age in the control cohort of 271 patients was 50 (range, 19-67) years. In this cohort, 33 patients (12%) had myelofibrosis, 143 (53%) had AML, and 95 (35%) had MDS.

Cyclophosphamide pharmacokinetics

Peak plasma concentrations and AUC of CY and its metabolites HCY and CEPM are summarized in Table 3 and Figure 1. Patients receiving CY/ ^{T}BU showed considerable variability in exposure to CY metabolites, including a 3.7-fold variation in AUC_{CY}, a 3.6-fold variation in AUC_{HCY}, and a 4.8-fold variation in AUC_{CEPM}. Pharmacokinetic parameters for patients receiving CY/^{T}BU were compared to those previously obtained in 75

patients receiving ^{T}BU/CY conditioning [18]. Given the age-dependent pharmacokinetics of CY [22], these analyses were adjusted for patient age. The median age of the CY/^{T}BU cohort was 55 (range, 30–65) years, while the median age of the historical ^{T}BU/CY cohort was 44 (range, 20–66) years [18].

The sequence of CY/^{T}BU administration markedly affected CY pharmacokinetics (Table 3; Figure 1). When CY was given first (CY/^{T}BU), there was a significant increase in AUC_{CY} (4899 vs. 2563 μ(•h, $p<0.0001$) and a significant decrease in AUC_{HCY} (168 vs. 290 μ(•h, $p<0.0001$) compared to values with standard ^{T}BU/CY. There was also a trend toward reduced AUC_{CEPM} with CY/^{T}BU (475 vs. 522 μU•h, $p=0.14$). In the CY/^{T}BU cohort, there were no apparent differences in BU Css or in the AUC of CY and its metabolites between patients with myelofibrosis and those with AML/MDS (data not shown). In the CY/^{T}BU cohort, the association of the AUC of CY, HCY, and CEPM with SOS could not be evaluated statistically, since only two cases of SOS occurred. Relapse and NRM were not associated with the AUC of CY and its metabolites. However, higher AUC_{HCY} and AUC_{CEPM} were associated with inferior overall survival ($p=0.03$ and 0.02, respectively; Table 4).

Clinical outcomes in cases

All patients in the CY/^{T}BU cohort initially engrafted (defined by a rise in absolute neutrophil counts to >500 cells/μL for at least three consecutive days) at a median of 17 (range, 11–30) days after HCT. One patient with AML/MDS who received an HLA-allele-mismatched allograft from an unrelated donor suffered late graft failure three months after HCT.

Approximately half of cases (26/51, 51%) did not require parenteral nutrition in the first 20 days after allogeneic HCT. Among patients with myelofibrosis, the median peak serum total bilirubin through day +20 was 2.3 (range, 0.7–30.0) mg/dL. Among patients with AML/MDS, the median peak serum total bilirubin through day +20 was 1.1 (range, 0.5–12.4) mg/dL. The incidence of SOS was 0/20 (0%) in patients with myelofibrosis, and 2/31 (6.5%) in patients with AML/MDS (Table 5). No patient in the CY/^{T}BU cohort developed severe SOS.

Acute GVHD grades II–IV and grades III–IV occurred in 67% and 8% of cases, respectively, at a median of 28 (range, 8–102) days after HCT. Chronic GVHD developed in 41% of cases at a median of 189 (range, 92–530) days after HCT.

The median follow-up of surviving cases was 19 months, and 32 patients (63%) were alive at last follow-up. Day +100 mortality was 0% in patients with myelofibrosis and 3% in patients with AML/MDS. At two years after HCT, cumulative incidence estimates for overall survival were 68% in patients with myelofibrosis and 56% in patients with AML/MDS; NRM was estimated at 27% and 17% for patients with myelofibrosis and AML/MDS, respectively. The cumulative incidence of relapse was 11% in patients with myelofibrosis and 44% in patients with AML/MDS.

The major causes of death were relapsed malignancy in patients with AML/MDS, and GVHD (with or without concomitant infection) in patients with myelofibrosis. One patient with myelofibrosis died of metastatic prostate cancer, which was diagnosed approximately six months after HCT. One patient with AML/MDS committed suicide at day +102 after HCT.

Comparison of outcomes after CY/^{T}BU vs. ^{T}BU/CY

Among patients with myelofibrosis, CY/^{T}BU conditioning was associated with a significantly reduced incidence of SOS as compared to ^{T}BU/CY (0% vs. 30%, p=0.006). In patients with AML/MDS, rates of SOS were 6.5% with CY/^{T}BU and 9.2% with ^{T}BU/CY (p=0.61). There were no cases of severe SOS in the CY/^{T}BU cohort, compared to 11 in the ^{T}BU/CY cohort.

In patients with myelofibrosis, median peak serum total bilirubin levels through day +20 did not differ significantly by conditioning-agent sequence (2.3 mg/dL in the CY/^{T}BU group vs. 2.2 mg/dL in the ^{T}BU/CY group, p=0.95). In patients with AML/MDS, the CY/^{T}BU group showed a trend toward lower median peak serum total bilirubin levels through day +20 (1.1 mg/dL vs. 1.4 mg/dL, p=0.07).

Patients conditioned with CY/^{T}BU showed significantly lower day +100 mortality as compared to those conditioned with ^{T}BU/CY (2% vs. 12%, p=0.01). For patients with myelofibrosis, the two-year cumulative incidence of relapse was 11% with CY/^{T}BU and 6% with ^{T}BU/CY (p=0.62). There were no significant differences in the two-year cumulative incidences of NRM (27% vs. 25%, p=0.91) or overall survival (68% vs. 72%, p=0.78; Figure 2).

For patients with AML/MDS, the two-year cumulative incidences of relapse with CY/^{T}BU vs. ^{T}BU/CY were 44% vs. 20% (p=0.008); for NRM, 17% vs. 22% (p=0.84); and for overall survival, 56% vs. 64% (p=0.57; Figure 3). The higher incidence of relapse in AML/MDS patients conditioned with CY/^{T}BU remained statistically significant, albeit attenuated, after adjustment for higher disease risk in this cohort (unadjusted hazard ratio [HR] 2.57, p=0.008; adjusted HR 2.15, p=0.02).

DISCUSSION

The major findings of this study are: 1) daily IV BU can be safely administered following high-dose CY; 2) the sequence of administration of BU and CY substantially affects CY metabolism; and 3) CY/^{T}BU conditioning is associated with a significantly reduced risk of day +100 mortality, a substantially lower incidence of SOS, and an absence of severe SOS, compared to the standard sequence of ^{T}BU/CY. In patients with myelofibrosis, the reduction of SOS incidence from 30% to 0% with a simple reversal of conditioning-agent sequence is both statistically and clinically significant. Busulfan is not inherently hepatotoxic as a single agent *in vitro* or in human overdoses [6,23]. Our data reinforce the concept that regimen-related liver damage results largely from toxic metabolites of CY, although we recognize reports of hepatotoxicity attributed to BU in combination with fludarabine as well [24,25].

Recent reports support the safety of administered daily IV busulfan with cyclophosphamide [26,27]. However, Williams *et al*. concluded that daily IV BU at 3.2 mg/kg/day for four days followed by CY 60 mg/kg/day for two days resulted in excessive toxicity: autopsy-confirmed SOS occurred in two of the three patients who received this regimen and had a BU Css >1025 ng/mL [28]. Our cases received the same dose of CY, but in the reverse sequence, followed by daily IV BU at a higher initial dose of 4 mg/kg with subsequent BU doses targeted to a Css of 800–900 ng/mL. This target BU Css range is well below the BU Css ranges of 925–1025 ng/mL previously associated with elevated SOS rates in adults conditioned with BU/CY [2,29,30]. Our data demonstrate acceptable toxicity when CY is administered before targeted daily IV BU at an initial BU dose of 4 mg/kg. Notably, the clearance of daily IV BU did not change during days -5 to -3 in patients receiving CY/^{T}BU [17]. Nevertheless, even in patients receiving CY/^{T}BU, CY metabolism showed substantial

interpatient variability when the CY dose, phenytoin dose, and BU Css were held constant between patients (Table 3; Figure 1).

Patients receiving CY/^{T}BU had higher exposure to CY, lower exposure to HCY, and similar exposure to CEPM compared to ^{T}BU/CY-conditioned patients. These data agree with our previous report comparing CY/TBI (i.e., CY first) to ^{T}BU/CY (i.e., CY after busulfan/phenytoin) [18]. The use of phenytoin as a prophylactic antiepileptic may contribute to this difference. We sought to characterize the clinical significance of the variability in CY pharmacokinetics (Table 4). Reduced HCY exposure may theoretically translate into less immunosuppressive effect, although AUC_{HCY} was not associated with clinical outcomes in patients conditioned with CY/TBI or ^{T}BU/CY [7,18]. Regarding toxicity, we previously described AUC_{CEPM} as a reporter for liver toxicity, since it strongly correlates with sinusoidal hepatotoxicity and mortality [7,22]. A pharmacodynamic analysis of SOS with CY metabolites could not be conducted, because only two of the CY/^{T}BU cases developed SOS. There was a statistically significant relationship between overall survival and the AUC of HCY and CEPM (Table 4).

Altering the sequence of conditioning agents is an appealingly simple and inexpensive strategy which uses available, familiar medications. Following preclinical studies [5,31,32], this approach has been translated into clinical trials in humans. Kerbauy *et al*. described the use of CY/BU conditioning in a cohort of 11 patients and reported lower peak serum aminotransferase levels compared to BU/CY-conditioned historical controls [14]. Of note, peak serum total bilirubin levels were not significantly different. In a larger retrospective cohort of 59 patients conditioned with CY/BU, Cantoni *et al*. reported lower rates of SOS and transplant-related mortality as compared to a small historical cohort of 16 patients conditioned with BU/CY [15].

Our results extend these earlier retrospective reports in the form of a prospective clinical trial. In addition to prospective enrollment, novel aspects of this study include a focus on patients at high risk of hepatotoxicity (those with myelofibrosis), the availability of a large cohort of concurrent control patients conditioned with ^{T}BU/CY, the determination of CY pharmacokinetics, and pharmacokinetic BU targeting to rule out variability in BU exposure as a confounding factor. The target plasma busulfan Css was 800-900 ng/ml for 100% of cases (IV busulfan) and 96.7% of controls (PO and IV busulfan). IV BU has been associated with reduced hepatotoxicity compared to oral BU when dosed by body weight [33]. However, when BU dosing is personalized to a target steady-state concentration, as in our study, outcomes appear to be similar regardless of route of administration [26]. Thus, given the consistent pharmacokinetic targeting of BU in our case and control patients, the route of administration is unlikely to account for the observed differences in outcomes.

We observed a higher risk of relapse in patients with AML/MDS conditioned with CY/^{T}BU, as compared to concurrent AML/MDS patients conditioned with ^{T}BU/CY. Some of this risk may relate to confounding variables: patients at high baseline risk of relapse were over-represented in the case cohort, and the relapse rate in the control cohort (20%) was somewhat lower than that generally reported in the literature [34]. Nonetheless, we cannot rule out the possibility that reversing the conditioning-agent sequence may increase the risk of relapse in patients with AML/MDS. Thus, our data do not support the use of this regimen in AML/MDS outside the confines of a well-designed clinical trial.

The major limitation of our study is the use of a concurrent/historical control cohort rather than prospective randomization between CY/^{T}BU and ^{T}BU/CY. Our control cohort contained a higher proportion of patients receiving bone marrow (as opposed to G-PBMC) allografts. However, as the most recent available data suggest equivalent outcomes with

bone marrow vs. G-PBMC allografts [35], this discrepancy is unlikely to be a significant source of bias in terms of the clinical outcomes of interest. Similarly, our control cohort contained a larger number of patients with HLA-mismatched donors compared to our case cohort. However, after excluding patients with HLA-mismatched donors from both cohorts, CY/^{T}BU conditioning continued to be associated with a significantly lower incidence of SOS in myelofibrosis patients (0% vs. 28%, p=0.01) and lower day +100 mortality (2% vs. 12%, p=0.02), suggesting that our findings were not influenced by this imbalance in donor/recipient HLA matching.

In conclusion, the present data show that reversing the sequence of conditioning agents (from ^{T}BU/CY to CY/^{T}BU) before allogeneic HCT was associated with reductions in day +100 mortality and in the incidence of SOS in patients with myelofibrosis. This reduction in hepatotoxicity was likely mediated by reduced exposure to toxic CY metabolites. This change in conditioning sequence, which requires no additional institutional expertise and employs existing medications and technology, can substantially reduce regimen-related toxicity and early mortality and improve outcomes in patients undergoing allogeneic HCT for myelofibrosis.

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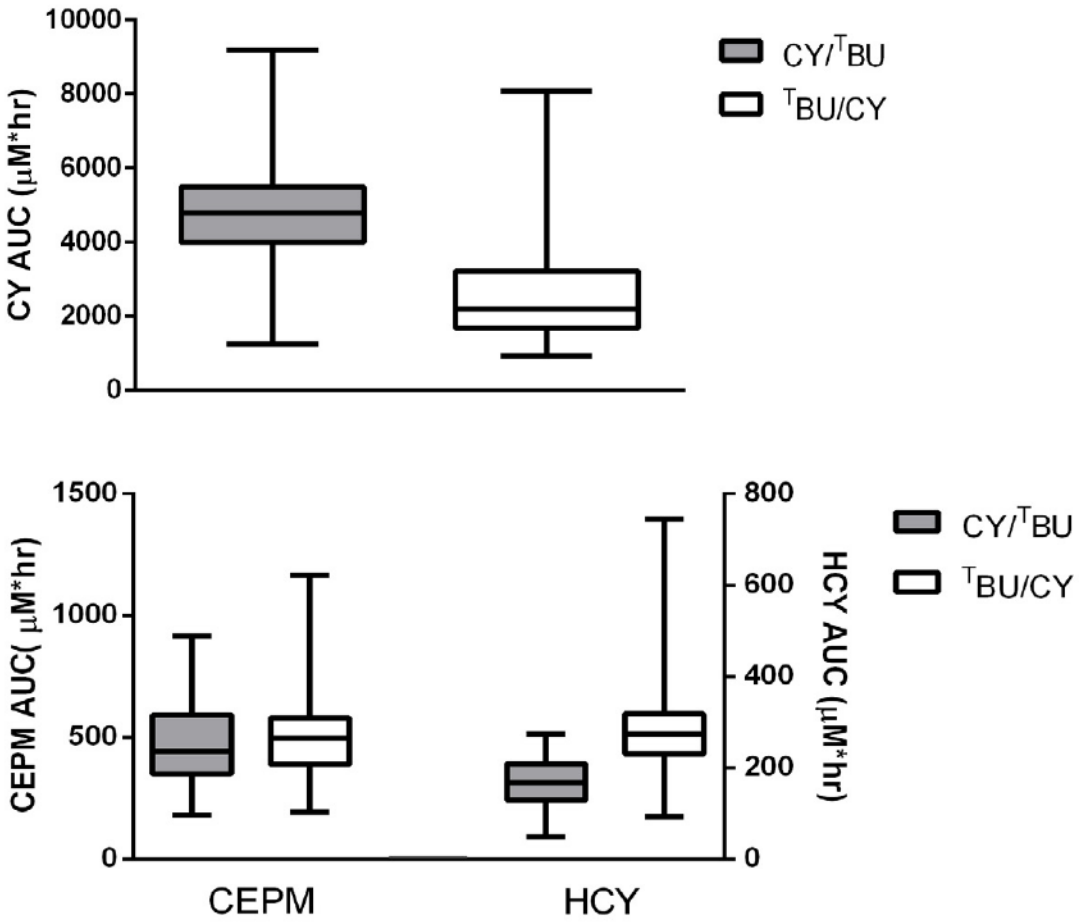
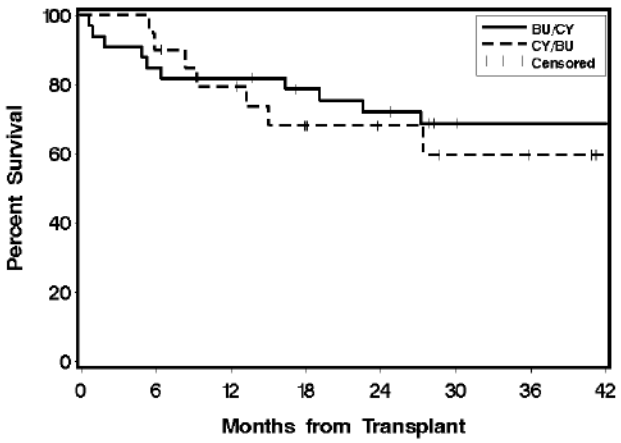
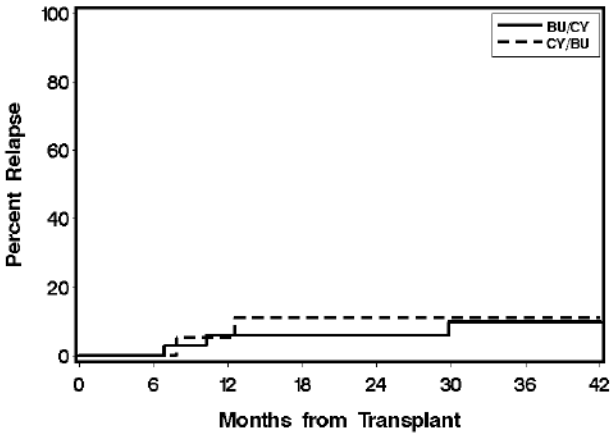


Figure 1.
Comparison of CY, HCY, and CEPM exposure by conditioning regimen. $AUC_{0\text{-}48hr}$ in patients receiving CY/^{T}BU (gray) and ^{T}BU/CY (white). Box designates 25th, 50th, and 75th percentile; whiskers designate 5th and 95th percentiles.





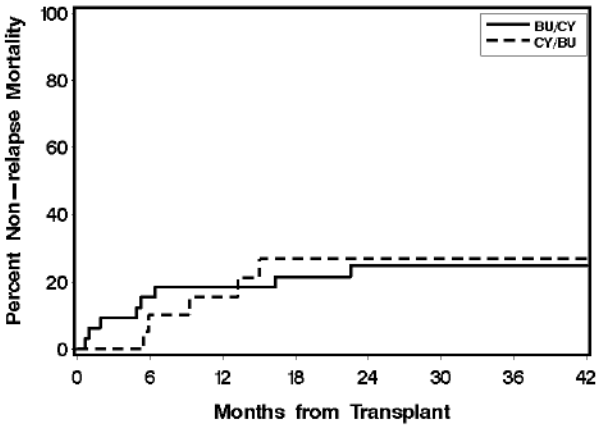


Figure 2.

Overall survival, non-relapse mortality, and relapse in patients with myelofibrosis conditioned with CY/^{T}BU (n=20) vs. ^{T}BU/CY (n=33). Abbreviations: CY, cyclophosphamide; ^{T}BU, targeted busulfan.

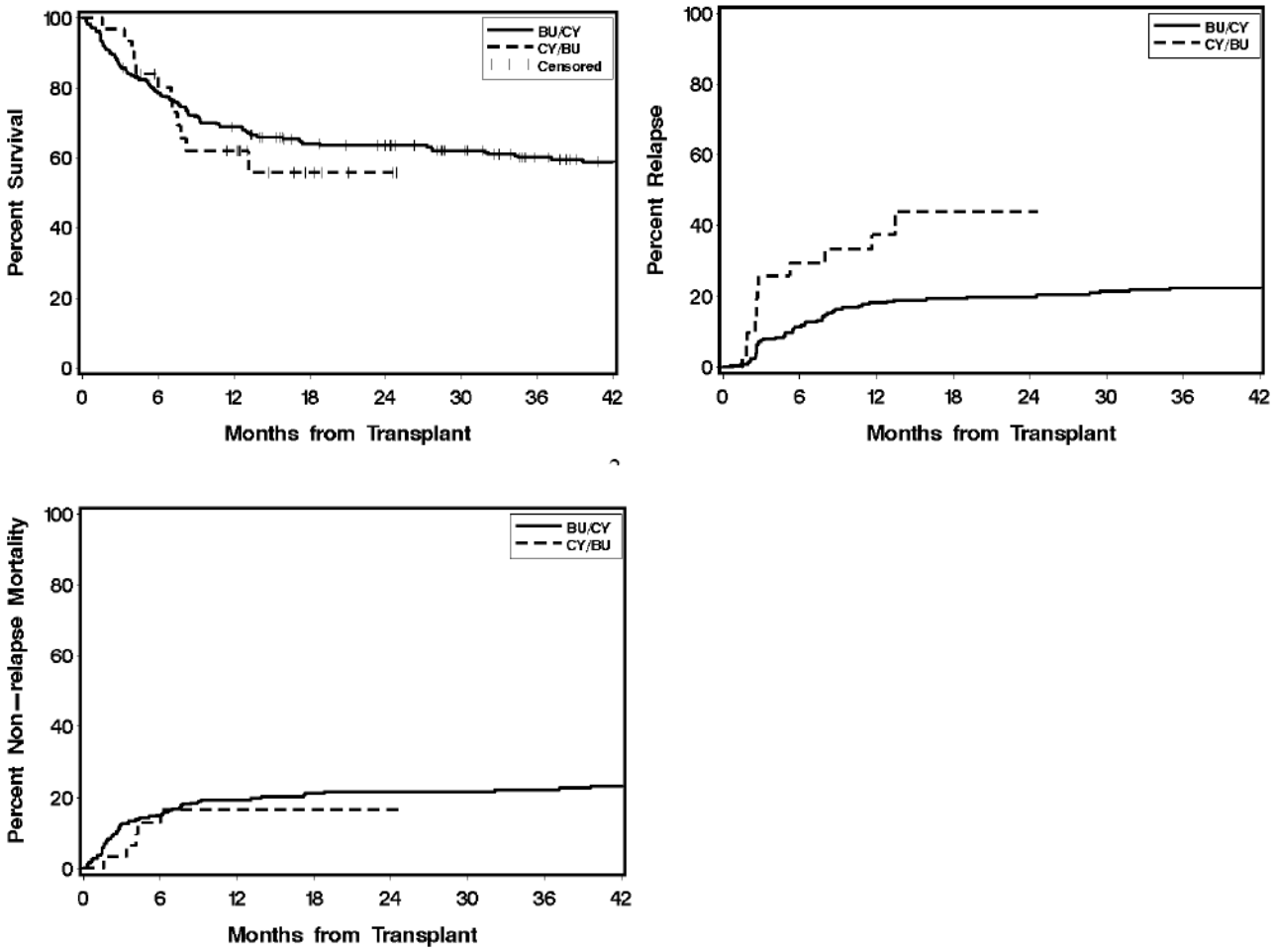


Figure 3.

Overall survival, non-relapse mortality, and relapse in patients with AML/MDS conditioned with CY/^{T}BU (n=31) vs. ^{T}BU/CY (n=238). Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CY, cyclophosphamide; ^{T}BU, targeted busulfan.

Table 1

Conditioning regimens for case (CY/^{T}BU) and control (^{T}BU/CY) patients.[a]

	CY/^{T}BU Cases (n=51)	^{T}BU/CY Controls (n=271)
Conditioning agents, by transplant day		
-7	CY IV 60 mg/kg[b]	BU[c]
-6	CY IV 60 mg/kg	^{T}BU
-5	BU[c]	^{T}BU
-4	^{T}BU	^{T}BU
-3	^{T}BU	CY IV 60 mg/kg[b]
-2	^{T}BU	CY IV 60 mg/kg
-1	Rest	Rest
0	Allograft infusion	Allograft infusion
Busulfan administration route & dosing frequency		
Oral every 6 hours	0	252 (93%)
IV every 6 hours	0	15 (6%)
IV once daily	51 (100%)	4 (1%)
Cumulative busulfan dose (mg)		
Oral	Not applicable	1048 (572–1916)
IV	1098 (580–1510)	976 (608–1668)
Target busulfan Css (ng/ml)		
≤900[d]	0	2 (0.7%)
600–900	0	3 (1.1%)
800–900	51 (100%)	262 (96.7%)
>900[d]	0	4 (1.5%)
Busulfan pharmacokinetics		
Dose #1 Css >900 ng/mL	23 (45%)	128 (47%)
Average daily Css[e] >900 ng/mL	1 (2%)	18 (7%)
Average daily Css[e] (ng/mL)	856 (811–1191)	861 (627–968)

[a] Data presented as median (range) or number (%). Abbreviations: ^{T}BU, targeted busulfan; CY, cyclophosphamide; IV, intravenous; Css, busulfan steady-state concentration.

[b] Mesna given concurrently with IV cyclophosphamide to prevent hemorrhagic cystitis.

[c] Phenytoin started one day before busulfan and continued throughout busulfan administration (i.e., day -6 through day -1 for CY/^{T}BU, and day -8 through day -3 for ^{T}BU/CY).

[d] Specific target Css detailed in the busulfan dosing and pharmacokinetics section of text.

[e] Cumulative over all 4 days of busulfan administration. For Css, each patient's busulfan Css over all 4 days was calculated, and then divided by 4 to provide the average daily Css.

Table 2

Patient characteristics.[a]

Characteristic	CY/^{T}BU (n=51) Cases	^{T}BU/CY (n=271) Controls
Age, in years	55 (30–65)	50 (19–67)
Diagnosis		
Acute myeloid leukemia	20 (39%)	143 (53%)
Myelodysplasia	11 (22%)	95 (35%)
Myelofibrosis	20 (39%)	33 (12%)
Donor		
Related	28 (55%)	96 (35%)
Unrelated	23 (45%)	175 (65%)
HLA-matched	21	98
1-HLA-allele-mismatched	2	77
Allograft source		
G-PBMC	51 (100%)	223 (83%)
Bone marrow	0 (0%)	48 (17%)
$CD34^{+}$ dose, in cells/kg recipient weight	13.4 (6.8–28.5)	9.1 (0.5–45.0)
Kahl disease risk[b]		
Low/moderate	30 (59%)	196 (72%)
High	21 (41%)	75 (28%)

[a] Data presented as median (range) or number (%).

Abbreviations: CY, cyclophosphamide; ^{T}BU, targeted busulfan; HLA, human leukocyte antigen; G-PBMC, granulocyte colony stimulating factor-mobilized peripheral blood mononuclear cells; IV, intravenous.

[b] Kahl disease risk measures risk of relapse after allogeneic hematopoietic cell transplantation [21].

Table 3

Comparison of pharmacokinetics of CY, HCY and CEPM by conditioning regimen. Peak concentrations (μM) are the highest concentration recorded on that day; AUC (μM•h) is from time 0 to 48 hours. Comparisons are adjusted for age.

	CY/^{T}BU	^{T}BU/CY [18]	P-value
CY			
Peak [CY], day 1	375 ± 60	312 ± 171	<0.0001
Peak [CY], day 2	329 ± 66	283 ± 124	<0.0001
AUC_{CY}	4899 ± 1255	2563 ± 1190	<0.0001
HCY			
Peak [HCY], day 1	9 ± 5	35 ± 18	<0.0001
Peak [HCY], day 2	20 ± 9	36 ± 13	<0.0001
AUC_{HCY}	168 ± 48	290 ± 98	<0.0001
CEPM			
Peak [CEPM], day 1	12 ± 6	27 ± 12	<0.0001
Peak [CEPM], day 2	26 ± 11	32 ± 29	0.25
AUC_{CEPM}	475 ± 180	522 ± 194	0.14

Abbreviations: CY, cyclophosphamide; ^{T}BU, targeted busulfan; AUC, area under the curve; HCY, 4-hydroxycyclophosphamide; CEPM, carboxyethylphosphoramide mustard.

Table 4

Relationship between exposure to CY and its metabolites and clinical outcomes among cases conditioned with CY/TBU.[a]

		Pharmacokinetic Parameters		
Clinical Outcome	**N[b]**	**AUC_{CY}**	**AUC_{HCY}**	**AUC_{CEPM}**
Non-relapse mortality	10	HR=1.15 (p=0.64)	HR=1.67 (p=0.11)	HR=1.40(p=0.25)
Relapse	9	HR=1.05 (p=0. 84)	HR=1.2 (p=0.53)	HR=1.53 (p=0.10)
Overall mortality	20	HR=1.28 (p=0.26)	HR=1.74 (p=0.03)	HR=1.67(p=0.02)

[a] AUC modeled as continuous linear variable, with hazard ratios for AUC_{CEPM} and AUC_{HCY} representing increase in hazard ratio (HR) associated with increase in AUC of 100 μM•h. Hazard ratios for AUC_{CY} represent increase in hazard associated with increase in AUC of 1000 μM•h. Hazard ratios adjusted for age at time of HCT, type of donor, and relapse risk.

[b] Number of events in cohort in 51 cases.

Abbreviations: CY, cyclophosphamide; TBU, targeted busulfan; HR, hazard ratio; AUC, area under the curve; HCY, 4-hydroxycyclophosphamide; CEPM, carboxyethylphosphoramide mustard.

Table 5

Incidences of sinusoidal obstruction syndrome and liver disease of unknown etiology among patients conditioned with ^{T}BU/CY vs. CY/^{T}BU.

	CY/^{T}BU (n=51)	^{T}BU/CY (n=271)
Myelofibrosis	20	33
No liver disease	17 (85%)	19 (58%)
LDUE	3 (15%)	4 (12%)
SOS	0(0%)	10 (30%)
Mild	0	2
Moderate	0	6
Severe	0	2
AML/MDS	31	238
No liver disease	26 (84%)	203 (85%)
LDUE	3 (10%)	13 (5%)
SOS	2 (6%)	22 (9%)
Mild	0	3
Moderate	2	10
Severe	0	9

Abbreviations: CY, cyclophosphamide; ^{T}BU, targeted busulfan; LDUE, liver disease of unknown etiology; SOS, sinusoidal obstruction syndrome; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome.