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Phase I study of dose-escalated busulfan with fludarabine and alemtuzumab as conditioning for allogeneic hematopoietic stem cell transplant: reduced clearance at high doses and occurrence of late sinusoidal obstruction syndrome/veno-occlusive disease

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Abstract

Disease recurrence after allogeneic hematopoietic cell transplant (alloHCT) remains common, making improvements in conditioning regimens desirable. A dose-response relationship between busulfan exposure and outcome is known. Using individual real-time monitoring of the busulfan area under the curve (AUC), we aimed to determine the maximum-tolerated busulfan AUC in a conditioning regimen with fludarabine/alemtuzumab. Thirty-six patients with advanced hematologic malignancies were treated. Busulfan levels after a test dose and conditioning dose 1 allowed targeting of subsequent AUCs and dose-escalation above the starting AUC of 4800 $\mu\text{mol}\cdot\text{min}/\text{L}$. Clearance of busulfan test doses was not always sufficiently predictive of treatment dose AUC and, on average, test dose clearance was faster than treatment dose clearance. When the study was modified to use conditioning dose 1 pharmacokinetics instead, accurately targeted treatment AUCs were achieved, and dose-escalation was possible. Severe, late-occurring

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sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD) was the dose-limiting toxicity seen in 5/8 patients at an AUC level of 6800 $\mu\text{mol}\cdot\text{min}/\text{L}$. The risk for SOS/VOD correlated with the highest observed AUC (AUC_{max}) rather than with the average cumulative AUC (AUC_{avg}). Busulfan dose-escalation to a maximum-tolerated AUC of 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$ —higher than that achieved by current standard busulfan regimens—was accurate and achievable using real-time pharmacokinetics monitoring of the first conditioning dose. This AUC is now being studied in phase II for patients receiving busulfan/fludarabine/alemtuzumab as alloHCT conditioning.

Keywords

Clinical results; transplant toxicity; pharmacotherapeutics

Introduction

While allogeneic hematopoietic cell transplant (alloHCT) of patients with advanced hematologic malignancies has resulted in prolongation of life and occasional cures, rates of disease recurrence remain disappointingly high [1]. Since high-dose chemotherapy and alloHCT rely to a large degree on the principle of dose-intensity and dose-response [2], dose-escalation of chemotherapy represents the next logical step to improve disease recurrence.

Busulfan [1,4-bis-(methanesulfonyl)butane] is an alkylating agent used extensively in the conditioning of patients undergoing alloHCT [3,4]. Previous studies have indicated a dose-response relationship between busulfan exposure and outcome [5], and data suggest that the recurrence rate after alloHCT is inversely related to the busulfan plasma concentration [6]. However, the dose-limiting toxicity (DLT) of busulfan is liver toxicity, often presenting as sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD) [7]. Increased busulfan systemic exposure (area under the curve [AUC]) is associated with an increased risk for SOS/VOD [8,9], but SOS/VOD risk is also influenced by other medications [10], particularly cyclophosphamide [11], and transplant procedures [12–14].

Using intravenous (IV) busulfan (3.2 mg/kg) and fludarabine (50 mg/m²) with cyclosporine/methotrexate-based graft-versus-host disease (GVHD) prophylaxis, Russell *et al.* [15] demonstrated that IV busulfan-based conditioning can be well tolerated, with consistent engraftment and good antitumor effect. Yet, even with exposure to a similar dose-per-kilogram body weight, up to two-fold variation in busulfan AUC was detected between patients [15].

Since busulfan has a narrow therapeutic margin, more accurate determination of the AUC is desirable. It is widely thought that busulfan pharmacokinetics is relatively linear across a variety of doses [16,17]. We therefore hypothesized that a test dose could be used to predict the treatment doses required to achieve desired AUCs. We hypothesized that such an individualized ‘real-time’ pharmacokinetics approach during conditioning would allow more accurate AUC targeting. Because of the absence of cyclophosphamide in the regimen and the absence of methotrexate use for GVHD prophylaxis, we predicted that escalation of the

target busulfan AUC beyond that achieved by the current standard dose (3.2 mg/kg) would be safely achievable. Here we report the results from our phase I study in which delayed SOS/VOD was found to be the dose-limiting toxicity.

Methods

Study participants

The study was conducted at The University of Chicago from March 2005 to June 2007 after Institutional Review Board approval in accordance with the Declaration of Helsinki. All patients provided written informed consent. Follow-up was continued through April 2010. Patients were eligible if they had advanced hematologic malignancies requiring alloHCT, a human leukocyte antigen-identical or one-antigen-mismatched sibling or unrelated donor, Karnofsky performance status $\geq 60\%$, serum bilirubin ≤ 2 mg/dL, and serum creatinine <1.5 times the upper limit of normal. Patients with active liver disease (defined as serologic evidence of prior exposure to hepatitis B or hepatitis C virus, or more than two-fold elevation of transaminases) were not eligible.

Chemotherapy administration

A busulfan test dose (0.5 mg/kg, using actual body weight) was administered over 3 h to all patients within 8 days prior to conditioning. On transplant day -7 through and including day -3 , fludarabine (25 mg/m²/day) and alemtuzumab (20 mg/day) were administered. On day -6 through and including day -3 , immediately after fludarabine, treatment doses of busulfan were given (Figure 1). Busulfan was administered through a dedicated IV line at a steady rate over 3 h. Busulfan was diluted in normal saline to a concentration of 0.5 mg/mL. At the end of 3 h, the infusion pump was stopped. The line was not flushed for concern that this might cause spikes in busulfan levels. To account for residual busulfan in the 19.6 mL of tubing that was discarded, 9.8 mg of busulfan had been added to every bag.

Supportive care

For seizure prophylaxis, clonazepam (1 mg) was administered on the days surrounding test dose administration and three times a day throughout conditioning (evening of day -7 through day -2) [18]. Acetaminophen was prohibited during busulfan administration. CYP3A inhibitors/inducers were avoided whenever possible, but if required (e.g. azole antifungals), care was taken not to change their dosing in the interval between test and treatment doses. Post-transplant immunosuppression consisted of IV tacrolimus beginning day -2 and then orally from engraftment until at least day 100. Use of prophylactic ursodiol was left to the discretion of the treating physician. Other supportive care procedures were as previously described [19].

Enrollment group 1: test dose-based AUC targeting

In an initial group of 21 patients, conditioning doses of busulfan were calculated by comparing the observed test dose AUC with the target AUC using:

$$\text{Conditioning dose (mg)} = [\text{target AUC} \times \text{test dose (mg)}] / \text{test dose AUC}$$

In cases where test dose AUC could not be calculated using a first-order elimination method, a standard busulfan dose of 3.2 mg/kg/day [15] (using actual body weight) was used.

Pharmacokinetics blood samples were taken immediately before busulfan infusion (t0), at 5 min before completion (t175), and at 1 h (t240), 2 h (t300), 3 h (t360), and 4 h (t480) after completion using a variation of the method of Vaughan *et al.* [20]. At every blood draw, approximately 8 mL of blood was aspirated through a dedicated peripheral line. This was immediately spun down and the sample forwarded to the laboratory. Assays for nearly all of these initial 21 patients were performed in the Pharmacology Core of The University of Chicago Cancer Research Center, based on the methods of Bostrom *et al.* [21]. The assay is linear from 70 to 7912 ng/mL, with inter- and intra-day precisions of <10%. The program WinNonLin Pro (Pharsight Inc., Mountain View, CA) was used for calculating AUC based on a single-compartment, first-order elimination model.

Dose-escalation design—We employed the modified continual reassessment method to determine the maximum-tolerated AUC [22,23]. Cohorts of size $n = 3$ were utilized, and we targeted the 25th percentile of the tolerance distribution, i.e. the AUC level producing DLT in 25% of the population. After the first cohort of three patients had been followed for a minimum of 21 days post-transplant, the observed number of DLTs was used to update the posterior distribution and determine the succeeding cohort. In enrollment group 1, if an additional patient required transplant prior to the completion of 21 days' follow-up for a given three-patient cohort, the cohort was expanded with additional patients at the current AUC level. Therefore, sometimes four or five patients were treated at the same level before dose-escalation occurred. After the observation of late-occurring DLT, the study was modified for all subsequently enrolled patients (enrollment group 2, described further below), and at least 60 days of follow-up in each cohort of at least three patients was required before dose-escalation was allowed; cohorts were again expanded if necessary. The treatment AUC levels began at level 0 = 4800 $\mu\text{mol}\cdot\text{min}/\text{L}/\text{dose}$, and each successive AUC enrollment level represented a 1000 $\mu\text{mol}\cdot\text{min}/\text{L}/\text{dose}$ increase if <25% of patients had DLT.

Enrollment group 2: AUC targeting using dose 1 AUC

When it became apparent that the test dose did not always predict for actual measured dose 1 AUC and that delayed SOS/VOD was dose-limiting, changes were made in the (1) AUC targeting method and (2) the pharmacokinetics laboratory. Also, as mentioned above, for safety purposes, dose-escalation to the next dose level was not permitted until 60 days of follow-up was observed in all patients at the existing dose level. Fifteen patients were accrued in this second enrollment group, starting again at dose level 0.

In group 2, although a test dose was administered and its kinetics documented, the test dose was not used for the calculation of treatment doses. Busulfan conditioning doses 1 and 2 were 3.2 mg/kg (using actual body weight). Dose 3 was re-targeted based upon dose 1 pharmacokinetics:

$$\begin{aligned}\text{Target AUC dose 3} &= (\text{target average AUC} \times 2) - (\text{AUC}_{\text{observed dose 1}}) \\ \text{Dose 3 (mg)} &= [\text{dose 1 (mg)} \times \text{target AUC dose 3}] / \text{AUC}_{\text{observed dose 1}}\end{aligned}$$

Dose 4 was identical to dose 3. Doses 3 and 4 were targeted so that the average AUC over all 4 days of busulfan equaled the target AUC for the enrollment level.

The use of dose 1 AUC to adjust doses 3 and 4 required the use of a laboratory that could provide rapid turnaround of samples. The samples for patients in enrollment group 2 were therefore sent to Mayo Clinical Laboratories (Toxicology and Drug Monitoring Laboratory, Mayo Clinic, Rochester, MN). Their method of quantitation used a modification of an assay by Vassal *et al.* [24]: 200 μ L of standards, controls, and samples were mixed with 50 μ L of internal standard (IS; busulfan-d4 2.0 μ g/mL in methanol), 750 μ L of 3.0 mol/L sodium iodide, and 1.0 mL of ethyl acetate. The vials were incubated for 45 min at 70°C with continuous agitation; the organic layer was dried under nitrogen and reconstituted in 75 μ L of ethyl acetate. One microliter was injected on an Agilent 6890/5973 gas chromatograph/mass spectrometer (GC/MS) using a DB-5MS, 15 m, 0.25 mm I.D. capillary column with a film thickness of 1 μ m. The initial temperature of 60°C was increased over 1.5 min to 150°C, which was maintained until the end of the analysis at 6.25 min. Single ion monitoring was used for the detection of busulfan (183 m/z) and IS (187 m/z). Busulfan concentrations were calculated by comparison of peak-height ratio of the drug to IS using a five-point standard curve. The assay is linear from 25 to 3000 ng/mL, with inter- and intra-day precisions of <10%. The serum was frozen as soon as possible following collection. Again, the program WinNonLin Pro (Pharsight Inc., Mountain View, CA) was used for calculating AUC based on a single-compartment, first-order elimination model.

Toxicity monitoring

Toxicity was scored according to National Cancer Institute/Common Toxicity Criteria (NCI/CTC) version 3 (<http://ctep.cancer.gov/reporting/ctc.html>). Any grade 5 toxicity was considered a DLT, and grade 4 toxicities were considered DLT with the exception of grade 4 hematologic toxicities, grade 4 infections, or grade 4 toxicities attributed to infection. Diagnosis of SOS/VOD was based on the pentad of weight gain, ascites, right upper quadrant abdominal pain, hepatosplenomegaly, and liver function analysis [7]. Whenever possible, the diagnosis was confirmed by liver biopsy with pathologic examinations being performed by an expert liver pathologist (J.H.). Patients from both enrollment groups were included in all toxicity analyses.

Statistical analysis

Comparisons between groups were analyzed using Student's *t*-test. *p*-Values are two-sided and not adjusted for multiple comparisons. For comparison of clearance of the test dose with that of treatment doses, the Bland–Altman method was used [25,26]. The limits of agreement were defined as $\pm 10\%$. The analysis on agreement was restricted to patients in enrollment group 2.

Results

Patient characteristics

Thirty-six patients were treated, having the clinical characteristics shown in Table I.

Test dose clearance is not an accurate predictor of treatment AUC, but 'real-time' targeting based upon dose 1 pharmacokinetics results in accurate average AUCs

Using a test dose for predicting treatment doses assumes that clearances are not affected by dose. Remarkably, in seven of the 21 patients in enrollment group 1, test dose elimination did not follow first-order elimination pharmacokinetics. We were unable to identify an exact cause of this discrepancy despite a careful review of all concomitant medications, administration, and sample times. Instead of using other non-compartmental models, those patients were given a standard dose of busulfan. For the other 14 patients in the initial enrollment group, the relation between target AUC and median measured AUC is shown in Table II (left side). While not shown in Table II, it was interesting to find that the measured AUCs after treatment dose 1 for patients in enrollment group 1 who received the standard 3.2 mg/kg dose were indeed similar to the dose 1 measured AUCs of the patients in enrollment group 2 (who all received the 3.2 mg/kg dose for treatment dose 1): group 1 median = 5124 $\mu\text{mol}\cdot\text{min}/\text{L}$ (range: 3070–6452); and group 2 median = 5137 $\mu\text{mol}\cdot\text{min}/\text{L}$ (range: 2865–7943).

Since we could not identify the causes of poor modeling and of the large variations in AUC among the first enrollment group, we restricted further pharmacokinetics analyses to patients in enrollment group 2. The real-time pharmacokinetics targeting method used in enrollment group 2 (using treatment dose 1 pharmacokinetics to target doses 3 and 4) resulted in generally excellent achievement of desired treatment AUCs (Table II, right side). The average treatment dose clearance (0.164 L/kg $\cdot$ h, or 106 mL/min $\cdot$ m² assuming an average 70 kg individual with a body surface area of 1.8 m²) agrees well with the findings previously published for IV busulfan (109 mL/min $\cdot$ m²) [27].

Although the test dose was not used in enrollment group 2 for treatment dosing purposes, test dose pharmacokinetics in these latter 15 patients all followed first-order elimination, and there was a highly significant correlation between test dose and treatment dose clearances and between clearance on successive treatment days (correlation coefficient [r] = 0.72, and p = 0.002, for all comparisons). Nevertheless, despite the high degree of correlation, significant clearance differences were detectable: median busulfan clearance after test doses was 0.174 L/kg $\cdot$ h (coefficient of variation [CV] 26%), compared to 0.160 L/kg $\cdot$ h (CV 24%) for dose 1 (p = 0.002) and 0.157 L/kg $\cdot$ h (CV 23%) for dose 3 (p = 0.02 for comparison with test doses). There was no statistical difference between the clearances of treatment doses 1 and 3. As a means of further interrogating the lack of agreement between clearance of test doses and treatment doses in our study, we analyzed the percentage difference between test dose and average treatment dose clearances among patients in enrollment group 2 using a modification of the method by Bland and Altman [25]. Of the 15 patients, 10 had test dose clearance values that were more than 10% different from the measured treatment dose clearances (Figure 2). In each of these 10 patients, test dose clearance was an overestimate of the actual treatment dose clearance. The average disagreement between test dose and treatment dose clearance was +15% for the group, including three patients whose test dose clearance values were more than +30% different from the treatment dose measurements. The more rapid clearance of the test dose compared to that of treatment doses translated to a significantly shorter plasma half-life of the test dose. The volumes of distribution of test and

treatment doses were usually very similar (data not shown). Disagreements between test dose and treatment dose clearance were even more pronounced in the first enrollment cohort (data not shown).

These data suggest that, despite excellent correlation, clearance of the pre-conditioning test doses was a poor predictor of clearance of the conditioning doses in our study. Achievement of desired average treatment AUCs was, however, possible by instead using a method of real-time targeting based upon pharmacokinetics data from the first busulfan treatment dose.

Finally, although again patients in enrollment group 2 were not dosed based upon their test dose pharmacokinetics results, for comparison purposes we analyzed whether dosing would have been significantly different in this group had test dose data been used. The overall actual administered busulfan doses ranged from 100 to 651 mg in the dose 1-strategy (group 2) portion of the study, compared to a range of 181–727 mg in the test dose-strategy (group 1) portion of the study. We found that a test dose-based strategy would have resulted in a non-significant average 12% increase in the administered busulfan dose among patients in enrollment group 2 compared to the employed strategy of using the pharmacokinetics data from treatment dose 1. Four of the 15 (group 2) patients' doses would not have been substantially different (different by >10%) using the two methods, while four other patients would have received substantially lower doses using a test dose-strategy. Perhaps importantly, six group 2 patients would have received substantially higher doses if a test-dose strategy had been used, and four of these six would have received doses that were more than 35% higher.

Busulfan dose-escalation

Overall, eight patients were treated at AUC level 0 (4800 $\mu\text{mol}\cdot\text{min}/\text{L}$), 13 at level 1 (5800 $\mu\text{mol}\cdot\text{min}/\text{L}$), and eight at level 2 (6800 $\mu\text{mol}\cdot\text{min}/\text{L}$). Level 3 was not reached. The pre-study plan of enrolling three patients per AUC level was routinely exceeded because of expansion of cohorts and enrollment of additional patients (see 'Methods').

In enrollment group 1, four patients were treated at AUC level 0, eight at level 1, and two at level 2. Seven others in enrollment group 1 received a 'non-adjusted' flat dose of 3.2 mg/kg/day because of the inability to calculate test dose AUC.

In enrollment group 2, four patients were treated at AUC level 0, five at level 1, and six at level 2.

Early treatment-related toxicity

Early treatment-related morbidity was minimal. In particular, there was no excessive sedation with clonazepam and no seizures occurred. Engraftment occurred promptly in all patients and there were no cases of early or delayed pulmonary toxicity. No patients died before day 21. Four patients died from treatment-related causes between day 21 and day 100, including two from SOS/VOD, one from grade IV gastrointestinal GVHD, and one from progressive multifocal leukoencephalopathy. Four patients died before day 100 from disease progression. Two of them also had severe SOS/VOD. Finally, using long-term

follow-up, the overall rate of occurrence of any GVHD (acute or chronic) was 27.8% in our study population.

Dose-limiting toxicity: delayed SOS/VOD of the liver

The DLT was SOS/VOD of the liver, occurring in eight patients in total, five of whom were treated at dose level 2 (target AUC = 6800 $\mu\text{mol}\cdot\text{min}/\text{L}$) (Table III). Contrary to the usual presentation, SOS/VOD occurred late in our study population, at a median time to diagnosis post-transplant of 52 days (range: 33–77 days). The clinical, laboratory, and radiographic features that led to the diagnosis of SOS/VOD in all eight patients were carefully reviewed and included all the classical features. Additionally, seven of the eight diagnoses of SOS/VOD were confirmed by the identification of classic histopathologic findings on liver biopsy (five patients) or at autopsy (two patients). Other risk factors for SOS/VOD (age, donor type, human leukocyte antigen [HLA] mismatching, and active disease) were not different among those developing SOS/VOD compared to those who did not. Four cases of SOS/VOD were fatal (three at AUC level 2, and one using the standard flat dose). Two of the four fatalities occurred in patients with simultaneous disease recurrence, including one patient who had, on autopsy, in addition to centrilobular necrosis, involvement of the liver by lymphoma. Four patients recovered, though one of the four had persistent portal hypertension and repeated gastrointestinal bleeds.

Given these data, the maximum-tolerated target average AUC was 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$.

Busulfan pharmacokinetics and SOS/VOD risk

Using the actual measured AUC (average of treatment dose 1 and dose 3, termed the AUC_{avg}) for each patient, we found that no patients with a measured AUC_{avg} of <4800 $\mu\text{mol}\cdot\text{min}/\text{L}$ exhibited SOS/VOD (six patients fell into this range). One patient (out of 13) with AUC_{avg} between 4800 and 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$ experienced SOS/VOD. Four patients out of nine with AUC_{avg} between 5800 and 6800 $\mu\text{mol}\cdot\text{min}/\text{L}$ had SOS/VOD (all four had an AUC_{avg} above 6200 $\mu\text{mol}\cdot\text{min}/\text{L}$). Three patients out of seven with AUC_{avg} of >6800 $\mu\text{mol}\cdot\text{min}/\text{L}$ developed SOS/VOD. In the patients exhibiting SOS/VOD, the actual measured levels were as follows: patient 7 (target AUC 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$) had an actual treatment AUC_{avg} of 6436 $\mu\text{mol}\cdot\text{min}/\text{L}$; for patient 12 (target 6800) it was 16 286; for patient 14 (3.2 mg/kg/day) it was 9960; for patient 19 (3.2 mg/kg/day) it was 4922; for patient 31 (target 6800) it was 6718; for patient 33 (target 6800) it was 6610; for patient 35 (target 6800) it was 7707; and for patient 36 (target 6800) it was 6256.

Considering all study patients, we then examined whether AUC_{avg} , highest measured AUC (AUC_{max} , maximum measured AUC after dose 1 or dose 3), or clearance (either dose 1 clearance, dose 3 clearance, or [dose 1 + dose 3] average clearance) correlated with the occurrence of SOS/VOD. AUC_{avg} was not correlated with risk of SOS/VOD, but patients exhibiting SOS/VOD had a higher AUC_{max} compared to those without SOS/VOD ($p = 0.07$). This provides preliminary evidence that high exposure on a particular day, rather than cumulative exposure, might be the main risk factor for SOS/VOD. Busulfan clearance measured at any time was not different among those experiencing SOS/VOD versus those

without (e.g. dose 1 clearance was 0.152 L/kg*h in patients with SOS/VOD versus 0.153 L/kg*h in those without, $p = 0.14$).

Discussion

Busulfan has been widely used in conditioning for alloHCT since the pioneering work of Santos and Tutschka [28]. Since then, efforts have continued to optimize busulfan exposure and reduce toxicity. Several investigators have reported that higher AUCs are associated with improved disease control and better engraftment, but higher risk for SOS/VOD [8,9]. More recently the M. D. Anderson group reported that the IV busulfan/fludarabine regimen had a very limited risk of SOS/VOD [27]. It was thought that the absence of cyclophosphamide, a hepatotoxin, explained the reduced risk for SOS/VOD after busulfan/fludarabine compared with busulfan/cyclophosphamide. Based on these data we hypothesized that further dose-escalation of busulfan in combination with fludarabine might be possible, particularly since at our center we use no post-transplant methotrexate and have a low incidence of acute and chronic GVHD due to the use of alemtuzumab. Our study used individualized dosing of IV busulfan based upon real-time busulfan pharmacokinetics measurements during conditioning to demonstrate accurate and safe busulfan dose-escalation to a target AUC of 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$.

We had originally hypothesized that a test dose of busulfan prior to treatment doses would allow calculation of an 'individualized' dose for each patient at a predictable, desired target AUC. Based on recommendations by Vaughan *et al.* [20], we calculated AUC using a first-order elimination pharmacokinetics model. Previous authors indeed suggested that busulfan pharmacokinetics are linear across a range of doses [15–17]. Despite extensive precautions to avoid drug interference and assure accurate infusion times, there were several cases where a first-order elimination model did not accurately describe the elimination of busulfan. It remains unclear why this occurred. We cannot exclude that inexperience of the transplant team contributed to some of the aberrant results, particularly since poor modeling was no longer observed in the second study group. Though trapezoidal methods of AUC determination may be less susceptible to modeling difficulties than the one-compartment model we utilized, such incongruencies have been observed regardless of method in 10–20% of patients, and have often been attributed to previously unknown drug interactions [29,30].

In our second enrollment group, using a variant of methods proposed by Andersson *et al.* [5], we adjusted treatment doses 3 and 4 based on the measured AUC of treatment dose 1, to achieve a desired overall average AUC. This method was quite accurate, and targeting at three different AUC levels was successful. Because we also collected test dose pharmacokinetics data in the second cohort, we were able to conduct an analysis to determine whether a test dose-targeting strategy would also have resulted in accurate AUCs in this group of patients. While we did find a highly significant correlation between test dose and treatment dose clearances, closer examination showed that a test dose-targeted AUC model would not have reliably resulted in treatment AUCs within 10% of predicted (66% of patients' test dose clearances were >10% different from measured treatment dose clearances). In over 25% of patients, treatment AUCs would have been more than 20% different from predicted due to reduced clearance of treatment doses compared to test doses.

This is not surprising, since close correlation between two tests does not at all represent agreement [25,26]. Additionally, correlation does not take into account the variation due to residual error, nor does correlation provide any measure of systematic differences. A better measure of agreement is given by the Bland–Altman method [25], which clearly illustrates the discrepancies between test doses and treatment doses in our study. We therefore conclude that the pharmacokinetics of a comparatively small busulfan test dose does not sufficiently accurately predict the pharmacokinetics of larger treatment doses. Interestingly, our data suggest that busulfan clearance is faster and the half-life shorter at lower doses. Though there are a number of explanations for this observation, it seems likely that saturation of busulfan clearance at very high doses may occur.

Since the time our study was originally designed, four other groups have investigated the correlation or the agreement between busulfan test dose and subsequent treatment dose pharmacokinetics [29–31]. Lindley *et al.* reported in 2004 a comparison of clearance of oral test dose with that of several treatment doses and found a lack of agreement [32]. More recently, Beri *et al.* [30] used a test dose to target treatment doses to an AUC of 4800 $\mu\text{mol}\cdot\text{min}/\text{L}$ and observed average AUCs of 4992 ± 1100 $\mu\text{mol}\cdot\text{min}/\text{L}$, concluding that, by using the test dose strategy, none of their patients were underdosed. But high AUCs over 6000 $\mu\text{mol}\cdot\text{min}/\text{L}$ were observed in four of 23 patients. They did not provide data on correlation or agreement between test dose clearance and treatment dose clearance. Russell *et al.* [31] found that test doses tended to have a faster clearance than treatment doses when given over the same time period, as in our study. Yet when all doses were administered at the same (mg/h) rate, test dose clearance was more predictive of treatment dose clearance (and AUC), with a correlation coefficient of 0.85 [31], similar to our results in enrollment group 2. Agreement was not investigated in this report. In all studies, unexpected clearance differences between test and treatment doses occurred in ~10–20% of patients. In no study has a test dose been sufficiently reliable as the only predictor for treatment dose AUC. Similarly, in most studies, there were occasional patients in whom AUC modeling was difficult and standard dosing was used, underscoring a limitation common to many attempts at individualized therapeutics [33].

We submit that use of our adapted method of AUC-targeting (real-time targeting based on the first conditioning dose) should receive greater consideration. An alternative would be to use a combination of test dose-guided dosing with subsequent modification based upon first treatment dose pharmacokinetics. Toward the goal of achieving dose-escalation above currently used doses—and guided by the hypothesis that higher busulfan AUCs could lead to improvements in disease response [6]—the maximum-tolerated AUC_{avg} of 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$ in this study is indeed higher than the AUCs corresponding to the standard dose of 3.2 mg/kg [15], and therefore deserves further testing in future clinical studies aimed at improving response.

Above an AUC_{avg} of 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$, we found an unexpectedly high incidence of SOS/VOD, which was associated with maximum busulfan exposure. The time course of this syndrome was highly unusual in that all cases occurred 5 or more weeks after transplant, though other clinical and the histological characteristics were typical. This late-occurring, rare complication had not been anticipated in the original design of the study [34]. The

original stringent stopping rules and routine expansion of cohorts were observed until individuals had been followed for 3 weeks. Unexpectedly, the DLT occurred well beyond this time limit, so that several patients had already been accrued to higher dose levels when the initial DLTs at lower levels were observed.

The high incidence of SOS/VOD was unexpected, since our study was based on the premise that the omission of cyclophosphamide in the conditioning regimen [11], the omission of post-transplant methotrexate [10], and the low incidence of GVHD after alemtuzumab-based conditioning [35] would all significantly reduce the risk for SOS/VOD, thus permitting busulfan dose-escalation. Additionally, SOS/VOD was observed despite the use of ursodiol prophylaxis in the majority of patients who developed SOS/VOD. A similar regimen of busulfan/fludarabine without cyclophosphamide, tested in 96 patients, was previously reported not to be associated with severe SOS/VOD [27]. Studies from Calgary similarly did not observe SOS/VOD [15,36], though AUCs over 6000 $\mu\text{mol}\cdot\text{min}/\text{L}$ were associated with increased treatment-related mortality, mostly attributed to GVHD [36]. One can only speculate about possible causes of the discrepancy between our results and those from Calgary and M. D. Anderson. It is unlikely that our use of alemtuzumab in the conditioning regimen caused a propensity for SOS/VOD, since the SOS/VOD incidence in another study using fludarabine/alemtuzumab/melphalan (with post-transplant tacrolimus) [1] was extremely low (three of 164 patients over >7 years) (van Besien, unpublished data). Since alemtuzumab clearly reduces the incidence and severity of GVHD [35], one possible explanation is that this may have uncovered SOS/VOD syndromes in our study which in other studies may have been 'masked' (or simply characterized) as GVHD, a commonly cited cause of treatment-related mortality and associated with high AUC levels in busulfan/fludarabine studies [36]. Of interest, Platzbecker *et al.* recently reported a high incidence of observed SOS/VOD after busulfan/fludarabine but attributed it to their use of everolimus as GVHD prophylaxis [37]. In their study, SOS/VOD often occurred late as well, a median of 32 days after transplant (range: 10–51 days). Lastly, our patients often had very advanced and refractory disease. Though few, if any, had received prior gemtuzumab ozogamicin, one cannot exclude that prior treatment and ongoing morbidity contributed to an increased risk for SOS/VOD.

Importantly, the design of our study may also have contributed to the risk of SOS/VOD. We targeted an AUC_{avg} (over the 4 days of conditioning). In many cases, the AUC_{max} —the highest or maximum AUC observed with a particular dose—was considerably higher than the target AUC. AUC_{avg} was not correlated with SOS/VOD risk, but AUC_{max} was. We submit that high peaks of plasma busulfan exposure, rather than total average exposure, are responsible for causing SOS/VOD. This would explain why two cases of SOS/VOD occurred among the seven patients receiving standard-dose busulfan (3.2 mg/kg). One of these two had a very high AUC_{max} (9960 $\mu\text{mol}\cdot\text{min}/\text{L}$, after dose 3). A means of avoiding such high-peaking doses may be the method devised by Wood *et al.* [38], who recently reported a continuous-infusion method of infusing busulfan. In their study, dose-escalation to an AUC of 7603 $\mu\text{mol}\cdot\text{min}/\text{L}$ was achievable without DLT [38]. The lack of toxicity compared to our regimen may be explained by the pharmacokinetics of a continuous infusion (delivering each 'dose' over 24 h, compared to 3 h as in our study), since

continuous infusion methods naturally avoid exposure peaks (and troughs) during the dosing period. Our hypothesis contrasts with the observations from the M. D. Anderson and Calgary groups discussed above. It requires further investigation, and only begins to address an important clinical issue in targeting busulfan, namely whether peak levels, single-dose AUCs, or cumulative AUCs are the major determinants of toxicity and efficacy. These issues may be partially addressed by a currently ongoing multicenter study of IV busulfan for autologous transplant.

We conclude that accurate dose-escalation of busulfan is achievable using real-time pharmacokinetics monitoring of treatment doses, but not by using solely test dosing. Dose-escalation beyond an AUC_{avg} of 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$ resulted in unacceptable DLT in the form of severe delayed SOS/VOD, and thus the safe AUC range of busulfan remains narrow even when combined with fludarabine. The substitution of cyclophosphamide by fludarabine may have resulted in the observed delay in the occurrence of SOS/VOD rather than in a reduction of its occurrence. A phase II study using fludarabine, alemtuzumab, and a targeted busulfan AUC of 5800 $\mu\text{mol}\cdot\text{min}/\text{L}$ is ongoing.

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Figure 1.

Treatment plan. Schema showing the timing of a busulfan test dose prior to conditioning, the administration of busulfan conditioning doses on days -6 through -3, the administration of fludarabine and alemtuzumab on days -7 through -3, and the timepoints of busulfan pharmacokinetics sampling. bu, intravenous busulfan; AUC, area under the curve.

Figure 2.

Clearance of a test dose is faster than, and not predictive of, subsequent conditioning doses.

For the second cohort, 10 of 15 patients had test dose clearances >10% different from measured treatment dose clearances; in each case test dose clearance overestimated treatment dose clearance. The average disagreement between test dose clearance and measured treatment dose clearance was +15% for the cohort (range: -6.8% to +50.9%).

Table I

Clinical characteristics of the 36 patients.

Clinical characteristic	Number of patients
Age (median)	55 years
Males	25 (69%)
Transplant type	
HLA-identical donor	29 (81%)
Related	21 (59%)
Unrelated	8 (22%)
Alternative donor	7 (19%)
Diagnosis	
CLL	4 (11%)
CML	2 (6%)
AML	16 (44%)
ALL	1 (3%)
NHL	4 (11%)
MDS	7 (19%)
Other*	2 (6%)
Disease status [†]	
Low risk	1 (3%)
Intermediate risk	7 (19%)
High risk	28 (78%)
Performance status [‡]	
0	25 (69%)
1	11 (31%)

* Includes one patient with mixed AML/ALL and one patient with T-ALL/lymphoblastic lymphoma.

[†] Classification according to the American Society for Blood and Marrow Transplantation (ASBMT) 2009 risk categories (www.asbmt.org).

[‡] Eastern Cooperative Oncology Group (ECOG) scale.

HLA, human leukocyte antigen; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndrome.

Table II

Accuracy of achieving the target AUC using pharmacokinetics of test dose versus using pharmacokinetics of conditioning dose 1.

AUC level	Target AUC (μmol-min/L)	Targeted using test dose			Targeted using conditioning dose 1		
		<i>n</i>	Median measured AUC* (95% CI) (μmol-min/L)	Number of patients within 10% of target	<i>n</i>	Median measured AUC* (95% CI) (μmol-min/L)	Number of patients within 10% of target
0	4800	4	4301 (3831–5317)	3	4	5197 (4742–5673)	2
1	5800	8	6492 (4578–8278)	1 [†]	5	5763 (5416–6266)	4 [†]
2	6800	2	13 265 (7890–19 635)	0	6	6691 (6271–7292)	5

* Based on the methods employed when using the test dose versus the first conditioning dose targeting strategy (see 'Methods' section in text), the median measured area under the curve (AUC) was calculated as the median of all available measured dose 1 and dose 3 AUCs for all patients within that AUC level (test dose group), or the median of all available average (dose 1 + dose 3) measured AUCs for patients within that AUC level (conditioning dose 1 group).

[†] Pharmacokinetics data were missing for one patient in each of these groups.

CI, confidence interval, represents 95% limits of these measurements.

Table III

Dose-limiting toxicity: sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD).

AUC level	Target AUC ($\mu\text{mol}\cdot\text{min}/\text{L}$)	Patients with SOS/VOD	Fatal cases of SOS/VOD
0	4800	0/8	0
1	5800	1/13	0
2	6800	5/8	3
Flat dose (3.2 mg/kg/day)	–	2/7	1

AUC, area under the curve.