Busulfan Systemic Exposure Relative to Regimen-Related Toxicity and Acute Graft-versus-Host Disease: Defining a Therapeutic Window for IV BuCy2 in Chronic Myelogenous Leukemia

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ABSTRACT
Complete bioavailability of IV busulfan (Bu) provides dose assurance by reducing the interdose and interpatient variability in Bu systemic exposure (Bu-SE) associated with the oral formulation. We hypothesized that Bu-SE, represented by the area under the plasma concentration versus time curve (AUC), would correlate with treatment outcome after allogeneic hematopoietic stem cell transplantation (HSCT) for chronic myelogenous leukemia (CML). Therefore, we analyzed the risk of death, incidence of regimen-related toxicity, and incidence of acute GVHD (aGVHD) as functions of the per dose IV Bu AUC in 36 CML patients who received a HSCT from an HLA-matched family donor after the IV BuCy2 regimen. Per-dose Bu AUCs were calculated for each subject using data obtained for doses 1, 5, 9, and 13. Toxicity was evaluated using the modified National Cancer Institute criteria. Because no patient developed veno-occlusive disease, increased serum bilirubin was used to characterize hepatotoxicity. We found that the probabilities of developing gastrointestinal toxicity (P = .01), hepatotoxicity (P < .01), mucositis (P = .09), and aGVHD (P < .01) all increased with increasing AUC. Further, the risk of death was significantly lower for patients having a per-dose AUC between approximately 950 and 1520 µMol-min, whereas the risk increased sharply with either lower or higher AUC values. These data suggest that an optimal Bu therapeutic window, based on per-dose AUC, exists. Given the ability of IV Bu to provide a more consistent per-dose AUC, these results should be useful in designing future IV Bu-based treatment protocols for stem cell transplantation.

KEY WORDS
Busulfan • Systemic exposure • Stem cell transplantation • Acute graft-versus-host disease

INTRODUCTION
Allogeneic hematopoietic stem cell transplantation (HSCT) is an established curative treatment for patients with chronic myeloid leukemia (CML) [1]. A recent randomized study suggested that long-term outcome was improved when cyclophosphamide (Cy) was combined with high-dose oral busulfan (Bu) rather than with total body irradiation in the pretransplantation conditioning regimen [2,3]. Although the oral BuCy2 regimen is generally well tolerated, it has been criticized for not being immunosuppressive enough to reproducibly permit engraftment, especially when partially mismatched or matched unrelated marrow donors are used [4,5]. Low Bu systemic exposure (Bu-SE), measured as Bu area under the plasma concentration versus time curve (AUC), has been correlated with increased risks of graft rejection and leukemic relapse [5,6]. Further, high Bu-SE has been associated with serious hepatic veno-occlusive disease (VOD) [7-11] and neurologic toxicity (grand mal seizures) [10,12]. These data have been derived from studies of Bu pharmacokinetics (PK) after oral drug administration, in which erratic intestinal absorption leads to highly variable bioavailability and
These results should be useful in designing future IV Bu optimal therapeutic window for a delivered Bu dose exists. With only 11 deaths among 36 patients, they suggest that an AUC was either below 950 or above 1520, the survival time and AUC data were used to define an optimal therapeutic window for Bu-SE expressed as a per-dose Bu AUC when IV Bu is used in the IV BuCy2 regimen prior to allogeneic HSCT. For the 26 patients who achieved Bu-SE inside this window, which is approximately 950 to 1520 µMol-min, a significantly lower death rate was documented than that of the 10 patients whose AUC was either below 950 or above 1520 µMol-min. Although these numerical values are based on a small sample with only 11 deaths among 36 patients, they suggest that an optimal therapeutic window for a delivered Bu dose exists. These results should be useful in designing future IV Bu-based treatment protocols for stem cell transplantation.

**PATIENTS AND METHODS**

**Eligibility Criteria**

Patients were required to have Ph chromosome-positive (Bcr/Abl-positive) CML, a physiological age between 15 and 55 years, Zubrod performance status < 2, normal (or with clinically nonsignificant deviations from normal) renal and hepatic function (serum creatinine ≤ 1.5 mg/100 mL, bilirubin ≤ 1.0 mg/100 mL, serum glutamic-pyruvic transaminase [SGPT] ≤ 3 × the upper normal limit), cardiac left ventricular ejection fraction ≥ 50%, pulmonary function tests including forced expiratory volume in 1 minute (FEV1.0) and diffusing capacity of lung for carbon monoxide (DLCO) ≥ 50% of predicted, negative serology for hepatitis B and human immunodeficiency virus, and an overall life expectancy of at least 12 weeks. Patients were also required to have either marrow or granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood progenitor cells from an HLA-matched related donor available. All patients gave written informed consent to treatment and PK studies in accordance with institutional guidelines.

**Preparatory Regimen**

The treatment was modified from that of Tutschka et al [17] and has been previously described [18]. IV Bu 0.8 mg/kg ideal body weight was given over 2 hours every 6 hours for 16 doses by controlled-rate infusion, followed by Cy 60 mg/kg IV over 1 hour on each of 2 consecutive days. After a day of rest, HSCT was performed. As an alternative to the fixed-dose Bu, later patients received Bu at 1.0 mg/kg IV over 2 hours for 2 doses while an initial estimate of Bu-SE (AUC) was made using first-dose Bu plasma-concentration versus time data. If needed, 1 PK-guided dose adjustment was performed at dose 3, and that dose was used for the remaining 13 doses of a 16-dose regimen to achieve a targeted AUC$_{ss}$ of 1250 µMol-min (±20%). Additional blood samples were drawn around doses 5 and/or 9 and 13 to confirm that the dose adjustment resulted in a Bu AUC within the targeted AUC interval. The IV Bu (Busulfex [busulfan] Injection, Orphan Medical, Minnetonka, MN) [15,16] was diluted in normal saline to 0.5 mg/mL and infused through a central venous catheter. Patients on the last protocol who received 1.0 mg/kg for 2 doses and who had significant overweight (>20% above ideal weight) were dosed according to adjusted ideal body weight. For patients with up to 120% of ideal body weight, the actual weight was used. For patients who weighed more than 120% of ideal weight, the difference between actual and ideal weight was multiplied by 0.5, and this difference was added to the ideal weight and used as the final dosing weight for the first 2 doses. From dose 3 on, the dosing was then based on PK parameters.

**Supportive Care**

Phenytoin was administered as seizure prophylaxis before and during Bu treatment in all patients. Mesna, antiemetics, blood components, and other supportive care measures were used per institutional guidelines. Recombinant G-CSF (5 µg/kg per day) was started on HSCT transplantation day +7 and continued until the absolute neutrophil count (ANC) exceeded 3.5 × 10⁹/L. The prophylaxis against GVHD was based on tacrolimus in combination with low-dose methotrexate [19].

**Evaluation of Therapy**

The clinical endpoints of the study included regimen-related toxicity (GI toxicity, hepatotoxicity, mucositis), engraftment, aGVHD, overall survival, and disease-free survival. Engraftment was defined as ANC ≥ 0.5 × 10⁹/L. Inflammatory cytogenetic, fluorescence in situ hybridization (FISH) (regarding sex mismatched donor-recipient pairs) and/or restriction fragment length polymorphism (RFLP) data were collected to support the clinical impression of engraftment. Clinical remission was defined as normalization of marrow morphology and peripheral blood counts, disappearance of the Ph-chromosome with conventional cytogenetic technique, and disappearance of the Bcr-Abl transcript according to reverse transcription–polymerase chain reaction on bone marrow samples obtained on days +30 and/or +100 after transplantation and thereafter as indicated. Relapse and progressive disease were defined by the first day of detection. Survival was defined by the day of death with the cause of death noted. Disease-free survival was defined as the time to death or first relapse from continuous clinical remission, with the times of relapse and death censored at last follow-up. During hospitalization, all patients were monitored daily for adverse events and hematologic parameters, and clinical chemistry parameters were evaluated at least twice weekly. After discharge and up to HSCT day +100, all patients were followed for treatment-related toxicity (weekly), for the quality of engraftment, and for relapse. After HSCT day +100, disease status and survival were followed at least quarterly for the first year and then at gradually increasing intervals.

Hepatic VOD was diagnosed based on clinical examination and laboratory findings [20]. The evaluation of other toxicities was done according to the modified National Cancer Institute (NCI) criteria.
Bu Pharmacokinetic Assessment and Prospective Dose Adjustment

Blood samples (10 mL) for Bu analysis were drawn in conjunction with the first, fifth, and/or ninth infusions at the following times: immediately prior to drug infusion; at 15, 30, and 45 minutes after the start of infusion; at 5 minutes before the end of infusion (peak; “end of infusion sample”); and at 15, 30, 60, 120, 180, and 240 minutes after the end of the infusion. In addition, a sample was taken immediately prior to the 13th infusion (“trough”) and 5 minutes prior to its completion (“peak”). Because Bu was administered via a central venous catheter, all blood samples for PK studies were collected from a peripheral IV line to avoid possible contamination caused by the proximity between the ports of the triple-lumen central venous catheter used for drug administration. The samples were collected and placed on ice. After centrifugation in a refrigerated centrifuge, the plasma was cryopreserved at −70°C until analysis with high-pressure chromatography as described [21]. The Bu peak concentrations (C_{max}) and the corresponding peak time (T_{max}) were observed values. The AUC per Bu dose was calculated by dividing the drug dose by the final Bu plasma clearance estimate. Parameters such as volume of distribution of the central compartment, elimination rate constant, and microconstants were estimated, and steady-state volume of distribution, half-lives, and clearance were calculated from the primary parameters. The pharmacokinetic modeling was performed using the ADAPT II Software program, Version 4.0 (BMRS, University of Southern California, Los Angeles, CA [22]). The final Bu plasma clearance was determined by modeling all (doses 1, 5, and/or 9, and 13) Bu plasma concentration versus time data.

Statistical Methods

Unadjusted survival probabilities were estimated using the method of Kaplan and Meier [23]. Unadjusted 2-group survival comparisons were made using the log-rank test [24]. The Cox proportional hazards regression model [25] was used to assess the ability of patient characteristics or treatment to predict survival, with goodness-of-fit assessed by using the lowess method of Cleveland [27]. The Cox proportional hazards regression model [25] was used to predict survival, with goodness-of-fit assessed by using the lowess method of Cleveland [27]. The Cox proportional hazards regression model [25] was used to predict survival, with goodness-of-fit assessed by using the lowess method of Cleveland [27]. The Cox proportional hazards regression model [25] was used to predict survival, with goodness-of-fit assessed by using the lowess method of Cleveland [27]. The Cox proportional hazards regression model [25] was used to predict survival, with goodness-of-fit assessed by using the lowess method of Cleveland [27]. The Cox proportional hazards regression model [25] was used to predict survival, with goodness-of-fit assessed by using the lowess method of Cleveland [27].

Adjusted this value by repeating the entire process for each of 1000 random permutations of the 36 AUC values, with the adjusted P value defined as the proportion of the 1000 tests having P < P^*. A lowess smooth [27] was used to determine the manner in which each serious adverse event (SAE) indicator (GI toxicity, mucositis, hepatotoxicity, aGVHD) varied as a function of AUC. Logistic regression [30] also was used to estimate the probability of each SAE function of AUC. All computations were carried out in Splus [30] using standard Splus functions and the Splus survival analysis package of Therneau [31].

RESULTS

Patients and Disease Characteristics

Thirty-six patients who consented to both treatment and PK studies (optional on the fixed-dose variant of the program) were treated between June 1996 and September 2001. Their demographics are summarized as follows. Median age was 37 years (range, 21-57 years). There were slightly more men than women enrolled (26/10). All patients had Philadelphia chromosome–positive (Bcr/Abllpositive) CML, and 32 were in first chronic phase (CP), whereas 4 were in accelerated phase (AP). Twenty-six patients received bone marrow, and 10 patients had a peripheral blood progenitor cell graft.

Toxicity

All patients received the IV BuCy2 regimen as prescribed, and in no case was the treatment discontinued or interrupted because of side effects. All adverse events were consistent with what had been reported with the use of the oral BuCy2 regimen [17] or of other pretransplantation conditioning regimens; the administration of IV Bu brought no new, unexpected toxicity. No grade 4 regimen-related toxicity was recorded, and we detected no central nervous system or lung toxicity among these patients.

Patients with organ-specific toxicity of the GI tract included 10 patients experiencing grade 2 mucositis and 7 patients experiencing grade 3 mucositis (47%), whereas 6 patients had grade 3 diarrhea (17%). In the absence of VOD, hepatotoxicity was evaluated as the maximum serum bilirubin recorded in the time interval up to day 30 posttransplantation: 3 patients developed grade 2 liver toxicity (bilirubin, 1.1-2.9 mg/100 mL) and 7 patients had grade 3 (bilirubin, 3.0-10 mg/100 mL). Aside from rapidly reversible increases in bilirubin (within 10 days), no other signs of serious liver toxicity were recorded, and no liver biopsies were performed.

Engraftment and Chimerism

All patients showed engraftment at a median of 12 days posttransplantation. The time to engraftment was not significantly different between patients who received marrow (median, 13 days; range, 11-20 days) and those who received peripheral blood progenitor cells (median, 12 days; range, 12-16 days). Median time to recovery to 20,000 platelets/µL was 19 days (range, 10-53 days). The development of donor-derived hematopoiesis was further documented by cytogenetic markers and RFLP studies. Informative data were available from all patients at 1 and/or 3 months posttransplantation.
All patients achieved 100% donor chimerism. No patient had autologous hematopoietic recovery documented, and no one reverted to host-derived hematopoiesis unless underlying leukemia recurred.

Graft-versus-Host Disease

aGVHD was documented in 12 patients (33%), of whom 4 had grade II and 8 had grade III disease. No one developed grade IV aGVHD. No patient died of GVHD prior to BMT day +100, but 8 patients (22%) died of chronic GVHD (cGVHD) or its secondary complications beyond BMT day +100.

Deaths

Eleven of the 36 patients died during the study. Eight, 4 of whom had AUC values >1521 Mol-min (see below), died of complications of cGVHD. The remaining 3 patients died of recurrent disease (AUCs of 816 [AP], 866 [CP], and 1061 [CP] µMol-min).

PK Steady-State Parameters

Complete PK profiles were assessed at Bu doses 1, 5, 9, and/or 13. Additionally, peak and trough drug concentrations were always obtained at dose 13 if a whole profile had been obtained at dose 9. The analyses were performed on blood samples obtained from all 36 patients entered in the protocols. At steady-state, the population median AUC was 1265 µMol-min (range, 816-1,905 µMol-min), the mean half-life (T_{1/2}) was 2.44 hours (range, 1.80-3.15 hours), and the mean Bu plasma clearance was 2.54 mL/min per kg (range, 2.1-3.4 mL/min per kg). Clearance and T_{1/2} estimates did not change substantially from dose 1 to steady state (dose 5 or 9). The last 11 patients received Bu at an initial dose of 1.0 mg/kg infused over 2 hours every 6 hours with an initial estimate of Bu AUC determined around the first dose. Doses 3 through 16 were adjusted as necessary to achieve a target per-dose AUC of 1250 µMol-min (±20%). Estimation of Bu plasma clearance and AUC were also obtained at doses 5 and 9 and/or 13 to assess the result of the respective dose adjustments. No further dose adjustments were performed after Bu dose 3, except in 1 patient whose clearance estimate changed dramatically from dose 1 to dose 5.

Figure 1. Per-dose Bu AUC estimates from the last 11 patients, who received 1.0 mg/kg doses infused over 2 hours every 6 hours with PK assessment at the first dose. Doses 3 through 16 were then individualized, with the aim of an AUC_{ss} of 1250 µMol-min (±20%). The PK assessments were repeated at doses 5 and 9 and/or 13 to assess the results of respective dose adjustments. No further dose adjustments were performed after Bu dose 3, except in 1 patient whose clearance estimate changed dramatically from dose 1 to dose 5.

PK and Regimen-Related Toxicity

There was a strong correlation between Bu-SE, quantified by Bu plasma AUC, and regimen-related SAEs. These SAEs included mucositis, GI toxicity, hepatotoxicity, and aGVHD. Estimation of Bu plasma clearance and AUC were also obtained at doses 5 and 9 and/or 13 to assess the result of the respective dose adjustments. No further dose adjustments were performed after Bu dose 3, except in 1 patient who unexpectedly had a significantly lower clearance calculated after dose 5, with a resulting high AUC (1680 µMol-min). All other patients reached the target window for dose 5, and all 11 patients were in the target window by the all-dose AUC estimates. The individual per-dose AUC measurements for doses 1 and 5 and the average all-dose AUC (dose 13) for each of these patients are shown in Figure 1.

Table 1. Distribution of SAEs

<table>
<thead>
<tr>
<th>Mucositis (%)</th>
<th>GI Toxicity (%)</th>
<th>Hepatotoxicity (%)</th>
<th>GVHD (%)</th>
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<tr>
<td>7 (19.4%)</td>
<td>6 (16.7%)</td>
<td>10 (27.8%)</td>
<td>12 (33.3%)</td>
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Figure 2 illustrates the relationship between AUC and each type of SAE and shows that the probability of each SAE, including the 3 toxicities and clinically significant aGVHD, increased with AUC.

PK, Survival, and Relapse-Free Survival

A Cox proportional hazards regression model was obtained by a step-down procedure beginning with the covariates age, sex, disease stage (chronic/accelerated), and AUC. Only AUC was a significant predictor of survival, with the final log hazard of death a quadratic function of log(AUC). The fitted model is summarized in Table 2.

The estimated hazard of death as a function of AUC, based on smoothing splines rather than the fitted Cox model, is illustrated in Figure 3. The observed 11 death times are marked on the hazard curve. All 36 times of death or censoring times are given by a rug plot along the horizontal axis, and a lowess smoothed bootstrap 95% confidence interval is given by dotted lines.

Repetition of the analysis for event-free survival, with the event defined as either death or disease progression, resulted in a qualitatively similar model (Table 3), but the regression of survival time on AUC was not statistically significant.

Based on the fitted Cox model for survival, one may predict the probability that a future patient will survive at least 3, 6, 9, or 12 months, as a function of AUC. These 4 predicted survival probabilities, with accompanying 95% confidence bands, are shown in Figure 4.

Both the fitted Cox model and the estimated hazard in Figure 3 suggest that there is an optimal interval of AUC values that on average yields longer survival time. The optimal interval, derived as described in Statistical Methods, runs from about 943 to 1521. The lower interval endpoint, 943, is the mean of the 2 consecutive data values 905 and 981, because there are no sample values between this pair. Consequently, if 943 were replaced by any number between 905 and 981, the resulting optimal AUC interval based on this data set would be identical. Similarly, the upper endpoint, 1521, is the mean of the 2 data values 1495 and 1547. Thus, the optimal interval is roughly 950 to 1520 based on these data. The adjusted P values of the log-rank tests comparing patient survival and relapse-free survival in the 2 intervals are .20 and .41, reflecting the fact that they are obtained from an intensive multiple testing procedure, as well as the small sample size.

Table 2. Fitted Cox Model for Survival

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<th>Variable</th>
<th>Coefficient</th>
<th>Standard Error</th>
<th>P</th>
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<tbody>
<tr>
<td>Log(AUC)</td>
<td>-129.01</td>
<td>63.99</td>
<td>.04</td>
</tr>
<tr>
<td>Log(AUC)^2</td>
<td>9.04</td>
<td>4.49</td>
<td>.04</td>
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The Kaplan–Meier curves of the estimated survival probabilities for patients with AUC values inside versus outside the optimal interval are given in Figure 5A for overall survival and in Figure 5B for disease-free survival. These analyses together suggest that an AUC interval in the range of approximately 950 to 1520 μMol-min is optimal for the use of IV Bu in the IV BuCy2 regimen.

DISCUSSION
Numerous trials have demonstrated that oral BuCy2 is an efficacious pretransplantation conditioning regimen. PK studies of oral Bu have yielded important correlates with clinical outcome: low suboptimal Bu blood levels have been connected with graft rejection and inadequate posttransplantation leukemia control [5,6], whereas a high Bu-SE correlated with hepatic and neurologic toxicity [7-13]. Vassal concluded in a review of oral Bu that interindividual variations in Bu-SE may be in the range of 10- to 20-fold when using a fixed-dose oral Bu regimen of 1.0 mg/kg [32]. This high variability is likely to influence both toxic and therapeutic effects and adversely affect the outcome of Bu-based treatment. Several investigators have advocated individualized Bu dosing based on PK parameters to overcome the interindividual variations in oral Bu bioavailability. It was assumed that this strategy would help to control regimen-related toxicity and increase the overall treatment safety [8,33,34]. Such an approach is intellectually appealing, but the problems connected with individualized dosing of a drug that has greatly variable interdose bioavailability present a significant challenge [13]. This challenge was demonstrated in several reports from PK-directed oral Bu dosing trials with the aim of arriving at AUC values within a predetermined therapeutic window [35-37]. The varying outcomes of these trials are likely due to, at least in part, the unpredictable interdose Bu bioavailability [10,13,14].

The development of an IV Bu formulation promised to overcome the bioavailability problem of oral Bu through more reliable dose assurance. In the current study, IV Bu was used at a fixed dose of 0.8 mg/kg per dose based on the PK similarity of this dose to an oral 1.0 mg/kg dose [21]. All 36 CML patients in our present trial received all of their scheduled IV Bu doses with good tolerance, without either VOD or serious neurological toxicity. The side effects experienced were well-described problems following various types of myeloablative conditioning therapy for HSCT, and there was no treatment-related mortality at 100 days. All patients achieved engraftment as documented with complete chimerism by RFLP studies, and no occurrence of secondary graft failure was recorded.

Previous studies performed by our group have demonstrated that Bu PK parameters such as clearance, AUC, and T\(_{1/2}\) at steady state after IV Bu [18,21] were similar to those published PK parameters obtained with the oral drug [9-13,21]. In patients with different types of hematologic malignancies, highly reproducible Bu PK parameters have

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<tbody>
<tr>
<td>Log(AUC)</td>
<td>-88.41</td>
<td>62.07</td>
<td>.15</td>
</tr>
<tr>
<td>Log(AUC)(^2)</td>
<td>6.18</td>
<td>4.36</td>
<td>.16</td>
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been documented after IV Bu drug delivery [18]. This finding is important because the ideal Bu-SE, according to the literature concerning orally administered Bu, should be confined within a narrow therapeutic window. Rather than relying on empiricism in determining the therapeutic range for IV Bu, we performed a step-wise model analysis based on the available PK information and the clinical data.

Simple graphical and logistic regression analyses demonstrated strong correlations between the AUC and both toxicity and aGVHD. The non–model-based estimate of the hazard of death and the Cox model–based estimated probability of surviving for 1 year after HSCT both suggested the presence of an optimal therapeutic range for high-dose Bu. These results suggest that precise Bu delivery may be even more important than previously thought, not only in relation to regimen-related toxicity, but also in the development of clinically significant aGVHD and for the likelihood of being alive beyond 1 year after HSCT. The determined per-dose Bu AUC range (943-1521 μMol-min) for patients was computed to give the largest survival advantage compared with the survival of patients having AUC values outside the range (P = .002). Although this optimal AUC range, or therapeutic window, is specific to the statistical method that we have used and this particular patient data set, which has 11 deaths in 36 patients, a general conclusion is that an optimal range of AUC values likely does exist. Moreover, once data become available on PK/AUC and survival time for future patients treated with IV Bu-based protocols, we anticipate that the optimal interval from those data will be numerically similar to that found here. In this context, it is important to remember that the numeric values arrived at here represent administration of Bu in a 16-dose regimen together with Cy. It is also important to recognize that although the PK information correlated with outcome, clinical disease stage did not. Thus, 2 of the 4 AP patients were below the therapeutic range at 816 μMol-min (died of progressive CML) and 905 Mol-min (died of cGVHD), whereas 2 AP patients are alive at the time of this writing in complete remission (AUCs of 1183 and 1486 μMol/min). A potential correlation between clinical stage and outcome as has been previously reported can neither be confirmed nor excluded because of the low number of patients beyond first CP in this study. If the regimen is changed, eg, once-daily IV Bu is combined with fludarabine or another agent [38,39], the numerical values for the daily Bu-SE assessed as AUC will change, and the substitution of Cy with fludarabine may further modify the therapeutic range.

Our data suggest that IV Bu administration in the IV BuCy2 regimen for CML patients is optimized if the patients are assured a Bu-SE within the pharmacokinetically determined therapeutic window. This goal can be realized by a PK-directed dosing approach as demonstrated by the PK data from the last 11 patients in this study.

ACKNOWLEDGMENTS

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**Figure 5.** After determining an optimal therapeutic AUC interval based on the survival and PK information, we compared the overall survival (A) and disease-free survival (B) for patients inside this interval (950-1520 µMol-min) to those of patients outside the interval. The differences in overall survival and disease-free survival between these groups are highly significant ($P = .002$ and $P = .01$, respectively).


