Overcoming the Response Plateau in Multiple Myeloma: A Novel Bortezomib-Based Strategy for Secondary Induction and High-Yield CD34+ Stem Cell Mobilization

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Abstract

Purpose: This phase II study evaluated bortezomib-based secondary induction and stem cell mobilization in 38 transplant-eligible patients with myeloma who had an incomplete and stalled response to, or had relapsed after, previous immunomodulatory drug-based induction.

Experimental Design: Patients received up to six 21-day cycles of bortezomib plus dexamethasone, with added liposomal doxorubicin for patients not achieving partial response or better by cycle 2 or very good partial response or better (≥VGPR) by cycle 4 (DoVeD), followed by bortezomib, high-dose cyclophosphamide, and filgrastim mobilization. Gene expression/signaling pathway analyses were conducted in purified CD34+ cells after bortezomib-based mobilization and compared against patients who received only filgrastim/cyclophosphamide. Plasma samples were similarly analyzed for quantification of associated protein markers.

Results: The response rate to DoVeD relative to the pre-DoVeD baseline was 61%, including 39% ≥VGPR. Deeper responses were achieved in 10 of 27 patients who received bortezomib-based mobilization; postmobilization response rate was 96%, including 48% ≥VGPR, relative to the pre-DoVeD baseline. Median CD34+ cell yield was 23.2 × 10⁶ cells/kg (median of 1 apheresis session). After a median follow-up of 46.6 months, median progression-free survival was 47.1 months from DoVeD initiation; 5-year overall survival rate was 76.4%. Grade ≥3 adverse events included thrombocytopenia (13%), hand–foot syndrome (11%), peripheral neuropathy (8%), and neutropenia (5%). Bortezomib-based mobilization was associated with modulated expression of genes involved in stem cell migration.

Conclusion: Bortezomib-based secondary induction and mobilization could represent an alternative strategy for elimination of tumor burden in immunomodulatory drug-resistant patients that does not impact stem cell yield.

Introduction

High-dose chemotherapy and stem cell transplant (HDT-SCT) for the front-line treatment of multiple myeloma has contributed to improved overall survival (OS; ref. 1) and remains a standard of care in eligible patients (2). Attainment of a very good partial response or better (≥VGPR) with pretransplant induction therapy is associated with improved long-term posttransplant outcomes in previously untreated patients with multiple myeloma, including prolonged progression-free survival (PFS), event-free survival, and OS (3, 4); therefore, induction therapy should aim to achieve as rapid and as deep a response as possible before transplant (2).

The incorporation of novel agents, such as the proteasome inhibitor bortezomib (Velcade) and the immunomodulatory drugs (IMiDs) thalidomide (Thalomid) and lenalidomide (Revlimid), into front-line pretransplant induction regimens has resulted in improved response rates and long-term outcomes compared with conventional induction approaches (5–7). A range of combination regimens have been evaluated in clinical trials (2, 8), with triplet regimens incorporating at least one novel agent showing superior efficacy to doublet regimens (2, 9). Several newer induction regimens,
Translational Relevance

This study showed substantial activity with sequential use of non–cross-resistant agents/regimens as induction in myeloma, with secondary induction with DoVeD (bortezomib–dexamethasone ± liposomal doxorubicin) resulting in further tumor burden reduction in patients following stalled or plateaued responses to primarily immunomodulatory drug–based induction. Furthermore, the approach of adding bortezomib to standard stem cell mobilization therapy (cylophosphamide and filgrastim) resulted in deeper responses, providing further evidence for the cytoreductive effect of bortezomib in combination with cylophosphamide. Notably, bortezomib-based mobilization was associated with very high CD34+ stem cell yields, suggesting that bortezomib may promote stem cell egress. Exploratory gene expression and Ingenuity Pathway Analysis revealed modulated expression of genes with roles in cell migration and associated with the canonical ephrin signaling pathway in patients receiving bortezomib-containing mobilization. These patients also showed significantly decreased plasma levels of CXCL12 and angiopoietin-1, which could potentially be useful as biomarkers of improved CD34+ mobilization in bortezomib-treated patients.

including bortezomib-dexamethasone (VD), lenalidomide low-dose dexamethasone (Rd), bortezomib-thalidomide-dexamethasone (VTD), and bortezomib-doxorubicin-dexamethasone (PAD), have been designated as U.S. National Comprehensive Cancer Network (NCCN) category 1 recommendations for transplant-eligible patients with multiple myeloma based on high-level evidence and uniform consensus (10). Alternative induction regimens, such as clarithromycin (Biaxin)-lenalidomide-dexamethasone (BiRD; ref. 11), bortezomib-lenalidomide-dexamethasone (RVD; ref. 12), RVD plus pegylated liposomal doxorubicin (DOXIL; RVDD; ref. 13), and bortezomib-cyclophosphamide-dexamethasone (VCD; refs. 14, 15) have also shown very promising activity in phase I/II studies (2, 8), but their efficacy remains to be confirmed in phase III trials.

While most patients will respond to these induction regimens, not all will achieve ≥VGPR, and further treatment options are required in this setting to maximize transplant outcomes. According to the Norton–Simon hypothesis (16), the sequential, dose-dense use of agents or regimens that are not cross-resistant may increase the proportion of patients achieving postinduction complete response (CR) or ≥VGPR, which may translate into improved PFS and OS. Bortezomib and the IMiDs have shown different but overlapping mechanisms of action (17, 18); therefore, the use of an IMiD-based regimen after initial bortezomib-based induction therapy, or vice-versa, may increase the proportion of patients achieving pretransplant ≥VGPR. Several clinical studies of sequential induction regimens in patients with previously untreated multiple myeloma, including bortezomib-liposomal doxorubicin-dexamethasone (VDD) → thalidomide-dexamethasone (TD; ref. 19), VCD → VTD (20), and vincristine-doxorubicin-dexamethasone (VAD) → VTD (21), have already reported encouraging efficacy.

Bortezomib does not negatively impact stem cell collection (22) and enhances the cytoreductive activity of alkylating agents (14, 15, 23–25). The addition of bortezomib to a granulocyte colony-stimulating factor (G-CSF) + cyclophosphamide stem cell mobilization regimen might therefore offer additional pretransplant cytoreduction while not interfering with potential stem cell yield. A stem cell yield supporting 2 SCTs ($8 \times 10^6$ to $10^7$ CD34+ cells/kg) is the collection goal recommended in 2009 by the International Myeloma Working Group (IMWG; ref. 26). Identifying induction therapies that are both effective and capable of achieving this goal are paramount; induction and mobilization therapy before HDT-SCT should therefore aim to maximize both cytoreduction and stem cell yields.

We assessed both the efficacy and the safety of secondary bortezomib-based induction with DoVeD (bortezomib + dexamethasone ± pegylated liposomal doxorubicin) as well as the impact of adding bortezomib to mobilization (bortezomib + cyclophosphamide + filgrastim) in patients with multiple myeloma who had a stalled incomplete response or were at first relapse after prior IMiD-based induction therapy. Gene expression profiling and cellular signaling pathway analyses were also conducted on CD34+ cells from patients with multiple myeloma in an attempt to understand the molecular effects of bortezomib on stem cell mobilization.

Materials and Methods

Patients

All patients aged 18 years or more with active multiple myeloma ($\geq 1.0$ g/dL serum M-protein; $\geq 0.1$ g/dL serum creatinine; $< 2.5$ mg/dL; and serum total bilirubin $< 2.0$ mg/dL; and/or measurable plasmacytomas, with evidence of multiple myeloma–related end-organ dysfunction) were eligible if they had Durie–Salmon stage II/III disease, had not received prior treatment with a proteasome inhibitor, and had first-line IMiD-containing induction therapy with a maximum response of partial response or less (≤PR) followed by a stalled response or plateau (defined as no significant change in M-protein level for 3 successive monthly assessments) to continued initial treatment. Patients were also eligible if they had relapsed (determined in accordance with IMWG criteria; ref. 27) following one previous IMiD-containing induction therapy regardless of initial treatment response. Other eligibility criteria included Karnofsky performance status (KPS) $\geq 70$%; absolute neutrophil count (ANC) $\geq 1,000$ cells/mm$^3$; platelet count $\geq 75,000$ cells/mm$^3$; aspartate/alanine aminotransferase $< 3.0 \times$ upper limit of normal (ULN); serum creatinine $< 2.5$ mg/dL; and serum total bilirubin $< 2.0$ mg/dL.
Exclusion criteria included history of grade ≥2 peripheral neuropathy (PN), as defined by National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0; a history of other malignancies (except for basal cell or squamous cell carcinoma of the skin or carcinoma in situ of the cervix or breast) unless disease free for ≥5 years; a history of active unstable angina, congestive heart disease, serious uncontrolled cardiac arrhythmia, or myocardial infarction within the previous 6 months, or New York Heart Association (NYHA) Class III/IV heart disease; and known HIV or hepatitis A, B, or C positivity, or active viral or bacterial infections.

**Study design**

This single-center, open-label, phase II study was conducted between July 2005 and April 2011. Patients received six 21-day induction cycles of DoVeD (bortezomib 1.3 mg/m² on days 1, 4, 8, and 11; dexamethasone 40 mg on days 1–4, 8–11, and 15–18; plus, if <PR after 2 cycles or <CR after 4 cycles, liposomal doxorubicin 30 mg/m² on day 4 for the remaining cycles), followed by one 21-day mobilization cycle with bortezomib (as above), high-dose cyclophosphamide (3 g/m² on day 8), and filgrastim (10 μg/kg/d for 10 consecutive days starting 24 hours after cyclophosphamide administration on day 9). There was no delay between induction cycle 6 and the subsequent stem cell mobilization cycle. All patients received prophylaxis during induction and mobilization with trimethoprim/sulfamethoxazole, acyclovir, and omeprazole.

The study was approved by the Institutional Review Board of Weill Cornell Medical College (New York, NY) and was conducted according to the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice. All patients provided written informed consent.

**Assessments**

Safety was monitored throughout the study and adverse events (AE) were graded by NCI-CTCAE version 3.0. Responses were assessed relative to pre-DoVeD induction baseline M-protein levels after every cycle, according to IMWG uniform response criteria (28). PFS and OS were measured from the beginning of DoVeD induction therapy. All patients received filgrastim for mobilization. Following quality control analysis, one sample from each group was removed after conducting a principal component analysis, leaving 5 and 3 samples, respectively. Levels of CXCL12 (SDF-1α) were compared in 6 and 7 patients, respectively, and angiopoietin-1 levels in plasma were compared between samples from 7 and 9 patients, respectively, who received or did not receive bortezomib during mobilization.

**Gene expression/signaling pathway/plasma analyses.** Analyzes were conducted in patients who underwent bortezomib-based mobilization in the present study and in a control cohort of patients (n = 13) who underwent non-bortezomib–based mobilization with filgrastim 10 μg/kg/d ± high-dose cyclophosphamide (3 g/m²) as part of their treatment at Weill Cornell Medical College. This control cohort comprised patients with Durie–Salmon stage II/III MM who had received prior induction therapy with bortezomib (n = 3), lenalidomide (n = 3), or bortezomib plus lenalidomide (n = 5). Two patients had not received any induction therapy. All patients received filgrastim for mobilization at least 1 month after completing induction.

The gene expression profiles of CD34+ stem cells enriched from leukapheresis product were compared between 6 and 4 evaluable patients, respectively, who either received or did not receive bortezomib during stem cell mobilization. Following quality control analysis, one sample from each group was removed after conducting a principal component analysis, leaving 5 and 3 samples, respectively. Levels of CXCL12 (SDF-1α) were compared in 6 and 7 patients, respectively, and angiopoietin-1 levels in plasma were compared between samples from 7 and 9 patients, respectively, who received or did not receive bortezomib during mobilization.

**Microarray gene expression analysis.** CD34+ cells were purified from cryopreserved leukapheresis product using a human CD34-positive selection kit (Stem Cell Technologies). The purity of the CD34+ isolate was assessed by flow cytometry using an anti-human CD34-PE antibody (BD Biosciences). Total RNA was isolated from the CD34+ cells using the RNeasy Mini kit from Qiagen. RNA yield and quality was assayed using a Nanodrop (Thermo Scientific) and the Agilent Bioanalyzer 2100 (Agilent). About 25 to 200 ng of total RNA was reverse-transcribed and amplified using the WT-Ovation Pico RNA Amplification System from NuGEN Technologies. Amplified cDNA (5 μg) was fragmented, labeled with biotin, and hybridized to Affymetrix HG-U133 plus two microarray chips (Agilent), which contained more than 47,000 transcripts, 38,500 well-characterized human genes, and more than 54,000 probe sets. Raw intensity (.cel) files were imported and preprocessed using the Robust Multi-Array Average (RMA) algorithm (29).

Chip data were imported into the GeneSpring GX 11.5 program (Agilent Technologies). Signal values <0.01 were set to 0.01, arrays were normalized to the 50th percentile, and individual genes normalized to the median. An unpaired, asymptotic t test (variances assumed equal) with P < 0.05 was conducted comparing the bortezomib and non-bortezomib mobilization groups followed by filtration for greater or less than 2.0-fold differences was applied to determine potential differential expression. Additional filters for pathway and biomarker analyses were applied (P < 0.0025, fold change 2.0) using IPA (Ingenuity Systems). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through the GEO series accession number...
Ninety-five percent confidence intervals (CI) were calculated to assess the precision of the obtained estimates. All analyses were conducted in SAS Version 9.3 (SAS Institute Inc.) and STATA Version 12.0 (StataCorp).

Results

Patients and treatment

A total of 38 patients who had reached a plateau or were in first relapse after primary induction therapy were enrolled and constituted the intent-to-treat (ITT) population. Patient baseline demographics and disease characteristics are summarized in Table 1. Briefly, patients had a median age of 61 years (range, 27–76), 55% were male, 97% had Durie–Salmon stage II/III disease, and 53% had International Staging System (ISS) disease stage II/III. Twenty-two (58%) patients had cytogenetic abnormalities; 7 (18%) patients had high-risk cytogenetics [del(17p), t(4;14), or t(14;16) by FISH]. More than half the patients had received initial induction therapy with lenalidomide + dexamethasone ± clarithromycin.

A diagram summarizing patient flow through the study is shown in Supplementary Fig. S1. Patients received a median of 6 cycles (range, 2–6) of DoVeD induction therapy. Per protocol, only patients who did not achieve PR by cycle 2 or VGPR by cycle 4 received liposomal doxorubicin. In total, 28 (74%) patients received liposomal doxorubicin, 16 patients from cycle 3, and 12 patients from cycle 5.

Response to DoVeD induction therapy and following mobilization

Responses to initial induction therapy are summarized in Table 1. All 38 patients were evaluable for response after DoVeD secondary induction. The overall best response rate (ORR, ≥PR, as measured by ≥50% reduction in M-protein) to DoVeD from post-primary induction (pre-DoVeD) baseline was 61% (n = 23), including 18% CR (n = 7) and 39% ≥VGPR (n = 15); in addition, 10 (26%) patients had stable disease (SD) and 5 (13%) had PD as best response (Supplementary Table S1).

Twenty-seven of the 38 patients elected to proceed directly to HDT-SCT and underwent bortezomib-based mobilization. Twenty-six of these 27 (96%) patients achieved a post-mobilization response of ≥PR relative to post-primary induction baseline, including 4 (15%) sCR, 3 (11%) CR, and 6 (22%) VGPR (48% ≥VGPR; Supplementary Table S1). This increased response rate post-mobilization reflected the fact that some patients continued to respond positively to treatment during bortezomib-cyclophosphamide–based mobilization. Six patients with SD after DoVeD induction improved to PR after mobilization; in addition, there was 1 VGPR-to-sCR transition and 3 CR-to-sCR transitions after mobilization. Ten patients underwent stem cell mobilization with non–bortezomib-based regimens; 6 patients were mobilized with filgrastim alone, and 4 were mobilized with cyclophosphamide + filgrastim. Of these 10 patients, 1 patient each achieved CR and VGPR, and 2 patients achieved PR post-mobilization (Supplementary Table S1). One patient was not mobilized during the study due to septic arthritis.

Quantitative real-time PCR. Total RNA (50 μg) from CD34+ cells was reverse transcribed into cDNA using a qScript cDNA supermix reagent from Quanta Bio-Sciences, Inc. according to the manufacturer’s protocol. The reaction mixture was diluted to 100 μL with distilled water and 3 μL of the diluted cDNA was amplified using gene-specific TaqMan probes and primers from Applied Biosystems. The following TaqMan gene expression assays (ABI Life Technologies) were used to carry out quantitative PCR (QPCR) on selected genes associated with significant differential gene expression and pathway analyses: AKT2 (Hs01086102_m1), CXCR4 (Hs00607978_s1), PAWR (Hs01088574_m1), RAC1 (Hs01902432_s1), SOD2 (Hs00167309_m1), RNF11 (Hs00702517_s1), PTP4A1 (Hs00743856_s1), PTK2 (Hs00178587_m1), and ANKRDS36B (Hs00743856_s1). Real-time QPCR was carried out in triplicate using the ABI 7900HT machine (Applied Biosystems). Comparative ΔCt analysis was conducted to determine the fold change in gene expression between bortezomib (n = 4) and non-bortezomib (n = 3–4) samples.

Quantification of CXCL12, angiopoietin-1, and interleukin 8 in plasma. Plasma samples were collected from patients who received bortezomib-based mobilization or from the non-bortezomib–based mobilization control cohort on the same day that leukapheresis product was harvested for CD34+ cell isolation. Blood samples were drawn into vacutainers containing K2-EDTA. Blood was centrifuged at 1,500 × g at 4°C for 10 minutes and plasma was removed and cryopreserved at −80°C. CXCL12 and angiopoietin-1 concentrations were subsequently quantified using enzyme immunoassay kits (R&D Systems) according to the manufacturer’s protocol and were based on the average of duplicate samples. Interleukin 8 (IL8) plasma levels were quantified using the mesoscale assay according to the manufacturer’s protocol.

Statistical analyses

The observed differences in gene expression between CD34+ stem cells collected from patients receiving bortezomib versus non-bortezomib mobilization were tested for significance using the Benjamini–Hochberg false discovery rate for multiple testing correction. A 2-tailed t test was conducted to test the significance of the difference in CXCL12 plasma levels, and a Mann–Whitney U test was conducted to determine the significance of differences in angiopoietin-1 and IL8 plasma levels between samples from patients receiving bortezomib versus non-bortezomib mobilization. Kaplan–Meier survival analysis was conducted to evaluate PFS and OS. Univariate Cox regression analysis was used to measure the association between patient characteristics and PFS. Multivariate Cox regression analysis could not be conducted because of the limited number of PFS events in the analysis. All P-values are 2-sided with statistical significance evaluated at the 0.05 α-level. Ninety-five percent confidence intervals (CI) were calculated to test the significance of the difference in PFS events in the analysis. All values are 2-sided with statistical significance evaluated at the 0.05 α-level. Ninety-five percent confidence intervals (CI) were calculated to test the significance of the difference in PFS events in the analysis.
individual patients' data are presented in Table 2. The median CD34+ stem cell yield for patients mobilized with non-bortezomib–based regimens was 10.72 × 10^6 cells/kg (range, 4.79 × 10^6 to 15.77 × 10^6). No statistical comparisons were planned or conducted between these groups.

Twenty-five of 27 (93%) patients who underwent bortezomib–based mobilization subsequently underwent SCT using standard melphalan 200 mg/m^2 conditioning. Following transplant, the median time to ANC recovery among these patients was 11 days (range, 10–18) and the median time to platelet recovery was 16 days (range, 11–24; Table 2). The median times to ANC and platelet recovery for patients who received non-bortezomib–based mobilization were 14 and 15 days, respectively. Of the 15 patients who underwent stem cell mobilization with non-bortezomib–containing regimens, 8 subsequently underwent SCT and 2 did not.

### Safety
All 38 (100%) patients completed at least one cycle of DoVeD; 10 (26%) patients received only bortezomib plus dexamethasone, without the addition of liposomal doxorubicin. Safety data are summarized in Supplementary Table S2. Thrombocytopenia and neutropenia were the most common grade ≥3 hematologic AEs, with rates of 13% and 5% during DoVeD induction and 85% and 96% during mobilization, respectively. Grade ≥3 febrile neutropenia was not observed during induction and was observed in only 1 (4%) patient during mobilization. The most common grade ≥3 nonhematologic AEs during induction were hand–foot syndrome (11%) and PN (8%). Diarrhea was the only grade ≥3 nonhematologic AE reported during mobilization in 1 (4%) patient. In total, 28 (74%) patients experienced PN during DoVeD induction, including 9 (24%) with grade 2 PN and 3 (8%) with grade ≥3 PN. During bortezomib-based mobilization, 6 (22%) patients experienced PN but no grade ≥3 PN was observed. No additional grade ≥3 PN events were observed following induction. No serious AEs (SAE) were reported during induction therapy.

### Outcomes
Seven of the 38 (18%) patients had died by the final data cutoff (April 15, 2011); the median duration of follow-up from the start of DoVeD induction (based on survivors) was 46.6 months (range, 6.4–68.8 months). In the ITT population, median PFS from the start of DoVeD induction was 47.1 months (95% CI, 26.2 months, upper limit not estimable); the 5-year PFS rate was 28.3% (95% CI, 8.6%–52.2%; Supplementary Fig. S2A). The median OS was not estimable; the 5-year OS rate was 52.2% (95% CI, 38.5%–65.8%; Supplementary Fig. S2B).

The effect of multiple patient characteristics (including myeloma type, β2-microglobulin level, creatinine level, disease stage, albumin level) on PFS was evaluated by univariate Cox regression analysis. None of the factors analyzed had any significant association with PFS.

### Stem cell collection and SCT
CD34+ stem cell yields greatly exceeded the study goal of 10 × 10^6/kg in 23 of 27 (85%) patients who received bortezomib-based mobilization, with a median yield of 23.2 × 10^6 cells/kg (range, 6.8 × 10^6 to 294.2 × 10^6) obtained within a median of 1 collection day (range, 1–5). Individual patients' data are presented in Table 2.
Univariate analysis of the factors potentially affecting OS, and multivariate analyses of PFS and OS, could not be conducted because of insufficient events.

Gene expression/signaling pathway analyses

In an attempt to elucidate an underlying mechanism for the high stem cell yields observed with bortezomib-containing mobilization, gene expression profiles of enriched CD34\(^+\) cells from bortezomib-mobilized patients in this study were compared with those from patients who received non-bortezomib mobilization. In total, 12,727 genes were differentially expressed (\(P < 0.05\)) between the 2 patient groups (data not shown). After filtering using a cutoff of \(P < 0.0025\), 997 genes were determined to be analysis-ready and used for IPA. The 10 most significantly up- or downregulated genes identified in microarray analysis of CD34\(^+\) cells (following pathway analysis filters) from patients receiving bortezomib- versus non-bortezomib-containing mobilization are summarized in Table 3. Nine genes were subsequently selected for validation by QPCR (Supplementary Table S3) due to exhibiting either high levels of differential expression between patients receiving bortezomib- and non-bortezomib–containing mobilization or an association with high ranking networks and pathways identified using IPA. The genes validated by QPCR included \(RAC1\), \(ANKRD36\), \(PTP4A1\), \(AKT2\), \(SOD2\), \(CXCR4\), \(PAWR\), \(PTK2\), and \(RNF11\). In general, the direction of fold change in QPCR data was consistent with the direction of change in the microarray data; 8 of 9 genes tested were in agreement with respect to up- or downregulation. The level of gene expression changes (fold change up or down) varied between the QPCR and microarray datasets (Supplementary Table S3).

### Table 2. CD34\(^+\) stem cell collection and hematologic recovery post-SCT, by patient, in patients receiving bortezomib-based mobilization

<table>
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<th>Patient</th>
<th>Number of collection days</th>
<th>Days from start of mobilization to start of collection</th>
<th>Total CD34(^+) stem cells collected ((\times 10^6/kg))</th>
<th>Total CD34(^+) stem cells infused ((\times 10^5/kg))</th>
<th>Days to ANC recovery (&gt;1.5 (\times 10^9/L))</th>
<th>Days to platelet recovery (&gt;20 (\times 10^9/L))</th>
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<td>3.96</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>16</td>
<td>32.8</td>
<td>7.92</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>20</td>
<td>11.5</td>
<td>5.63</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>15</td>
<td>29.4</td>
<td>8.2</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>27</td>
<td>3</td>
<td>18</td>
<td>27.2</td>
<td>10.2</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1 (1–5)</td>
<td>18 (15–24)</td>
<td>23.2 (6.8–294.2)</td>
<td>8.2 (3.4–17.7)</td>
<td>11 (10–18)</td>
<td>16 (11–24)</td>
</tr>
</tbody>
</table>

\(^a\)20.4 \(\times 10^6\) cells collected after 2 days.

\(^b\)86.3 \(\times 10^6\) cells collected after 1 day.

\(^c\)Patient opted to defer HDT-SCT until later.
was the highest differentially expressed gene between CD34+ cells from patients receiving bortezomib- and non-bortezomib–containing mobilization. The highest-ranked canonical pathways (determined using IPA software) were hypoxia signaling in the cardiovascular system, protein ubiquitination, ephrin receptor signaling, mitochondrial dysfunction, and actin nucleation by ARP-WASP.

Ephrin receptor signaling is known to play a role in cell migration (30) and can be differentially expressed in mobilized hematopoietic stem cells (31). To determine whether differential gene expression in these highest-ranked pathways could account for enhanced bortezomib-induced mobilization of CD34+ cells, the list of genes was filtered for those with known functional roles in cell migration. Nineteen genes with known roles in cell migration were...
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Figure 1. Top 5 canonical cell signaling pathways associated with genes differentially expressed in CD34+ cells between patients receiving bortezomib- and non-bortezomib–based mobilization. The left y-axis displays the −log (P), which is calculated in IPA using a right-tailed Fisher exact test. The right y-axis displays a ratio calculated using IPA and determined by the number of significant genes (P < 0.0025, fold change = 2.00) divided by the total number of genes that make up the canonical pathway.

The ORR observed with DoVeD secondary induction in this small patient population is notable in the context of previous phase II and III studies of this combination in previously untreated patients with multiple myeloma, which reported ORR rates of 78% to 95%, including 42% to 62% ≥VGPR (4–6, 34–36). Our results indicate that substantial activity with DoVeD-like regimens in previously untreated multiple myeloma may be retained despite patients having previously received IMiD-based primary induction. Although other studies have already reported encouraging activity with sequential induction approaches in previously untreated multiple myeloma (19–21), this study is unique in showing activity in patients who received bortezomib-based secondary induction after achieving maximum incomplete responses to primary induction with IMiD-based regimens.

Previous studies have shown an association between the achievement of ≥VGPR before transplant and improved survival outcomes (3, 4, 9). In this study, after a median follow-up of 46.6 months, median PFS was 47.1 months, the 3-year OS rate was 76.4%, and only 7 patients had died. These outcomes compare favorably with respect to previous reports (3), despite the population being preselected for resistance to IMiDs; however, interpretation is limited by differences between patients in the treatment course received.

Subsequent to DoVeD secondary induction, further cytoreduction was seen after a single cycle of stem cell
Table 4. Differential gene expression in CD34+ cells in the ephrin signaling pathway between patients receiving bortezomib- and non-bortezomib–based mobilization.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>P</th>
<th>Fold change</th>
<th>Location</th>
<th>Function</th>
<th>Movement/migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI1</td>
<td>Abl interactor 1</td>
<td>6.14E-04</td>
<td>7.903</td>
<td>Cytoplasm</td>
<td>Nucleotide binding</td>
<td>Subcellular movement</td>
</tr>
<tr>
<td>ACTR2</td>
<td>ARP2 actin-related protein 2 homolog (yeast)</td>
<td>2.33E-03</td>
<td>8.778</td>
<td>Plasma membrane</td>
<td>Actin/nucleotide binding</td>
<td>Subcellular movement</td>
</tr>
<tr>
<td>ARPC3</td>
<td>Actin-related protein 2/3 complex, subunit 3, 21kDa</td>
<td>2.35E-03</td>
<td>4.622</td>
<td>Cytoplasm</td>
<td>Protein binding</td>
<td>Increased migration of NIH3T3 and HEK293</td>
</tr>
<tr>
<td>CFL2</td>
<td>Cofilin 2 (muscle)</td>
<td>2.33E-03</td>
<td>6.201</td>
<td>Nucleus</td>
<td>Protein binding</td>
<td>Increased migration of vascular smooth muscle cells</td>
</tr>
<tr>
<td>GNAQ</td>
<td>Guanine nucleotide binding protein (G protein), q polypeptide</td>
<td>2.29E-03</td>
<td>6.116</td>
<td>Plasma membrane</td>
<td>Enzyme</td>
<td>Migration of HUVEC</td>
</tr>
<tr>
<td>GNAS</td>
<td>GNAS complex locus</td>
<td>1.86E-03</td>
<td>7.411</td>
<td>Plasma membrane</td>
<td>Enzyme</td>
<td>Migration of prostate cells</td>
</tr>
<tr>
<td>GNB1</td>
<td>Guanine nucleotide binding protein (G protein), beta 1</td>
<td>1.11E-03</td>
<td>4.562</td>
<td>Plasma membrane</td>
<td>Enzyme</td>
<td>Migration of smooth muscle cells, jurkat cells and huvecs, SDF1</td>
</tr>
<tr>
<td>GRIN1</td>
<td>Glutamate receptor, ionotopic, N-methyl D-aspartate 1</td>
<td>1.65E-03</td>
<td>-2.799</td>
<td>Plasma membrane</td>
<td>Ion channel</td>
<td>Migration of neurons</td>
</tr>
<tr>
<td>GRIN2C</td>
<td>Glutamate receptor, ionotopic, N-methyl D-aspartate 2C</td>
<td>1.46E-03</td>
<td>-3.081</td>
<td>Plasma membrane</td>
<td>Ion channel</td>
<td>Projection neurons</td>
</tr>
<tr>
<td>ITGB1</td>
<td>Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)</td>
<td>1.56E-03</td>
<td>9.561</td>
<td>Plasma membrane</td>
<td>Transmembrane receptor</td>
<td>Migration in cell lines includes B lymphocytes</td>
</tr>
<tr>
<td>MAP2K1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
<td>1.08E-03</td>
<td>5.886</td>
<td>Cytoplasm</td>
<td>Kinase</td>
<td>Migration in cell lines including B lymphocytes</td>
</tr>
<tr>
<td>MAPK1</td>
<td>Mitogen-activated protein kinase 1</td>
<td>1.59E-03</td>
<td>6.554</td>
<td>Cytoplasm</td>
<td>Kinase</td>
<td>Migration in cell lines including bone marrow cells</td>
</tr>
<tr>
<td>NCK2</td>
<td>NCK adaptor protein 2</td>
<td>2.24E-03</td>
<td>4.436</td>
<td>Cytoplasm</td>
<td>Kinase</td>
<td>None</td>
</tr>
<tr>
<td>PGF</td>
<td>Placental growth factor</td>
<td>1.62E-03</td>
<td>-2.301</td>
<td>Extracellular space</td>
<td>Growth factor</td>
<td>Decreased expression causes increased migration in cells including progenitor cells</td>
</tr>
<tr>
<td>RAC1</td>
<td>Ras-related C3 botulin toxin substrate 1</td>
<td>1.22E-03</td>
<td>19.450</td>
<td>Plasma membrane</td>
<td>Enzyme</td>
<td>Migration in cell lines</td>
</tr>
<tr>
<td>RAP1A</td>
<td>RAP1A, member of RAS oncogene family</td>
<td>1.86E-03</td>
<td>5.896</td>
<td>Cytoplasm</td>
<td>Enzyme</td>
<td>Migration in cell lines</td>
</tr>
<tr>
<td>RAP1B</td>
<td>RAP1B, member of RAS oncogene family</td>
<td>1.25E-03</td>
<td>3.227</td>
<td>Cytoplasm</td>
<td>Enzyme</td>
<td>Migration in cell lines</td>
</tr>
<tr>
<td>SOS2</td>
<td>Son of sevenless homolog 2 (Drosophila)</td>
<td>1.61E-03</td>
<td>4.056</td>
<td>Cytoplasm</td>
<td>Protein binding/catalytic domain</td>
<td>None</td>
</tr>
<tr>
<td>WIPF1</td>
<td>WAS/WASL interacting protein family, member 1</td>
<td>1.81E-03</td>
<td>6.030</td>
<td>Cytoplasm</td>
<td>Actin/protein binding</td>
<td>None</td>
</tr>
</tbody>
</table>
mobilization with bortezomib + cyclophosphamide + filgrastim, with 10 of 27 (37%) patients improving their quality of response compared with post-DoVeD induction, including some converting to sCR. These results add to a growing body of evidence showing the cytoreductive effect of bortezomib in combination with cyclophosphamide (14, 15, 23) and highlight the use of continued bortezomib treatment through mobilization.

The unexpected finding of enhanced CD34+ stem cell yields with the combination of bortezomib,
cyclophosphamide, and filgrastim for mobilization suggests that bortezomib may promote stem cell egress. This led us to undertake exploratory, retrospective gene expression analyses, and IPA in an attempt to try to understand the mechanism(s) underlying this phenomenon. We validated our microarray gene expression data using QPCR, a widely accepted tool for such validation. The results of our QPCR validation experiments indicated an overall agreement in the direction of gene expression changes when compared with microarray data but a variation in the level of fold change between the 2 methods, as well as reported in other studies (37, 38). Such inconsistencies are likely due to inherent differences between the 2 methods, differences in normalisation methods, and the limited amount of RNA available for QPCR (n = 3–4).

The upregulation of IL8 expression levels in CD34+ cells supports a role for IL8 signaling in stem cell mobilization, and this has been reported in the serum of normal human donors after G-CSF mobilization (39). IL8-induced stem cell mobilization has also been shown in mice (40). Here, we report that there are no significant changes in IL8 plasma protein levels between bortezomib- and non-bortezomib–mobilized patients suggesting that IL8 may play a role in both mobilization strategies. The significant increase in IL8 at the gene expression may be due to differences in expression levels found in CD34+ cells versus secreted IL8 protein levels detected in plasma. The mechanisms associated with IL8-induced stem cell mobilization remain to be elucidated.

Among the highest ranked canonical pathways exhibiting significant changes in gene expression identified in our analyses, the significant dysregulation of the ubiquitination pathway was an expected result given the use of bortezomib in one of mobilization regimens. Bortezomib prevents the ubiquitination of regulatory proteins involved in numerous pathways, including cell-cycle signaling, apoptosis, transcriptional regulation, and cell surface receptors and hypoxia-inducible factor (HIF)-α signaling.

Our analyses also revealed modulated expression of various genes known to have a functional role in cell migration and associated with the canonical ephrin signaling pathway in patients receiving bortezomib during mobilization versus a control cohort of patients not exposed to bortezomib during mobilization, thus implicating a role for ephrin signaling via CXCL12 and angiopoietin-1 modulation in the differential CD34+ cell yields observed in the 2 groups. The reason is unclear, but one potential explanation is that CXCL12 may be more reduced in bone marrow stromal cells than in circulating plasma, which would allow for CD34+ cell migration. A possible mechanism of action could be via the ephrin signaling pathway. Potentially, ephrin-positive hematopoietic progenitor cells (CD34+) interact with ephrin ligand–producing cells in the bone marrow stroma, resulting in detachment from the stromal cells and allowing the cells to migrate from the bone marrow. Similar mechanisms have been reported in cocultures of CD34+/ephrinB4+ cells with stromal cells expressing EphB2 ligand; in the presence of EphB2 ligand, CD34+ hematopoietic progenitor cells detached from the stroma (41). Ephrin receptors also exhibit bidirectional signaling mechanisms whereby ephrin proteins can both serve as ligands for ephrin receptors but also transduce afferent signals (upstream or downstream of signaling pathways) upon receptor binding (42). The ephrinB2 receptor and the expression of ephrinB2/Eph4 complexes have also been associated with the migration of endothelial cells via receptor-dependent and -independent mechanisms (30, 43). The downregulation or inhibition of CXCR4/CXCL12 signaling has been proposed as the mechanism by which stem cells exit the bone marrow microenvironment (44). As shown in Fig. 2B, the downregulation of this signaling axis in plasma is evident with bortezomib-containing mobilization. These findings are similar to those reported in a previous study in non-Hodgkin lymphoma (NHL) showing that CXCL12 is significantly decreased in patients with larger stem cell yields (45).

Another potential mechanism for bortezomib modulation of stem cell mobilization is via the downstream effects of proteasome inhibition on angiopoietin-1 expression (46). Angiopoietin-1 and its receptor, Tie2, are important for maintaining hematopoietic stem cell quiescence, as well as for adhesion of stem cells to the bone marrow microenvironment in mice (32). Previous studies have shown the ability of bortezomib to decrease angiopoietin expression in vitro models (47). In this study, we confirm that genes downstream of the angiopoietin signaling pathway were found to be differentially expressed in CD34+ stem cells from patients receiving bortezomib–versus non-bortezomib–containing mobilization. The binding of angiopoietin-1 to Tie2 may mediate migration of CD34+ cells and likely involves several kinases including phosphoinositide 3-kinase (PI3K), focal adhesion kinase (FAK), and PAK (48–50). This mechanism likely involves integrins. In our gene expression data, integrins, PI3K, and PAK were upregulated in bortezomib-treated versus non-bortezomib–treated patients. As with CXCL12, angiopoietin-1 produced from bone marrow stromal cells could be contributing to CD34+ cell migration from bone marrow and is likely to be differentially expressed in stromal cells.

In summary, this study has shown very promising response rates, high CD34+ stem cell yields, and manageable toxicity with bortezomib-based secondary induction in a relatively small multiple myeloma patient population who achieved a suboptimal response to predominantly IMiD-based primary induction therapy. Early identification of patients who achieve a suboptimal response to primary induction therapy may allow for a timely switch to a secondary induction regimen offering a greater chance of achieving better responses before transplant (4). Notably, the addition of bortezomib to mobilization or conditioning regimens to further enhance cytoreduction before SCT could be more broadly applicable; this agent is being investigated as a component of both, supported by preclinical data showing synergy with alkylating agents such as cyclophosphamide (in mobilization) and melphalan (in conditioning). Finally, our exploratory gene expression and pathway analyses have suggested the ephrin receptor and its...
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ligands as potentially of importance in CD34+ stem cell migration from the bone marrow, a finding that warrants further investigation.

Disclosure of Potential Conflicts of Interest
R. Niesvizky, T.M. Mark, and M. Coleman have served as consultants and participate as lecturers in the speakers’ bureaus for Celgene and Millennium Pharmaceuticals, Inc. R. Niesvizky received research support and drug for the implementation of this trial. D. Skerrett is a consultant/advisory board member of Mesoblast. K. Pekle has honoraria from Speakers Bureau of Millennium. F. Zafar is a consultant/advisory board member of Millennium Corp. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: R. Niesvizky
Expert patient care: R. Niesvizky

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Overcoming the Response Plateau in Multiple Myeloma: A Novel Bortezomib-Based Strategy for Secondary Induction and High-Yield CD34+ Stem Cell Mobilization

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