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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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Abstract

Purpose—This phase 2 study evaluated bortezomib-based secondary induction and stem cell mobilization in 38 transplant-eligible myeloma patients 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

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Conflict of interest disclosure:

R.N., T.M.M., and M.C. have served as consultants and participate as lecturers in the speakers' bureaus for Celgene and Millennium Pharmaceuticals, Inc. R.N. received research support and drug for the implementation of this trial. The other authors declare no competing financial interests.

Author contributions:

R.N. conceived the original idea and treatment program, wrote and implemented the protocol, provided patient care, coordinated the data and the database, coordinated input from all the authors, conceived the figures, and wrote the manuscript. T.M.M. implemented the protocol, provided expert patient care, and coordinated stem cell collection and transplant. M.W. conducted gene array and ELISA on samples, implemented correlatives, and helped on the correlative analysis. D.S.J. generated the database, coordinated translational efforts and marrow samples, and generated figures. R.N.P. provided significant input on trial design, interpretation of correlatives, and expert patient care. M.M. and J.S. coordinated the data and the database. P.J.C. performed biostatistical analysis. L.M. assisted with protocol generation and initiated the database. T.B.S. provided analysis of stem-cell collection and expert patient care. F.Z. and K.P. provided significant contributions to patient care. X.Z. implemented correlative analysis and gene array analysis. S.E. provided immunohistochemistry analysis of marrow. D.S. provided stem cell laboratory collection, cryopreservation, and characterization data, and interpretation. S.C.-K. coordinated translational research and gave direction in science. M.C. provided insight on the original idea and treatment program concept, helped with writing the manuscript, and provided expert care of patients. M.E.L. coordinated all correlative science, generated ELISA and Gene Array analyses in patients and controls, coordinated correlative discovery, and helped with writing the manuscript.

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 post-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; post-mobilization response rate was 96%, including 48% VGPR, relative to the pre-DoVeD baseline. Median CD34+ cell yield was 23.2×10^6 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.

Keywords

Bortezomib; CD34+; myeloma; secondary induction; stem cell mobilization

INTRODUCTION

High-dose chemotherapy and stem cell transplant (HDT-SCT) for the front-line treatment of multiple myeloma (MM) has contributed to improved overall survival (OS) (1), and remains a standard of care in eligible patients (2). Attainment of a very good partial response or better (VGPR) with pre-transplant induction therapy is associated with improved long-term post-transplant outcomes in previously untreated MM patients, 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 prior to 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 pre-transplant 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, including bortezomib-dexamethasone (VD), lenalidomide-low-dose dexamethasone (Rd), bortezomib-thalidomide-dexamethasone (VTD), and bortezomib-doxorubicin-dexamethasone (PAD), have been designated as United States National Comprehensive Cancer Network (NCCN) category 1 recommendations for transplant-eligible MM patients based on high-level evidence and uniform consensus (10). Alternative induction regimens, such as

clarithromycin (Biaxin[®])-lenalidomide-dexamethasone (BiRD) (11), bortezomib-lenalidomide-dexamethasone (RVD) (12), RVD plus pegylated liposomal doxorubicin (DOXIL[®]; RVDD) (13), and bortezomib-cyclophosphamide-dexamethasone (VCD) (14,15) have also shown very promising activity in phase 1/2 studies (2,8), but their efficacy remains to be confirmed in phase 3 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 post-induction complete response (CR) or VGPR, which may translate into improved PFS and OS. Bortezomib and the IMiDs have demonstrated 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 pre-transplant VGPR. Several clinical studies of sequential induction regimens in patients with previously untreated MM, including bortezomib-liposomal doxorubicin-dexamethasone (VDD) → thalidomide-dexamethasone (TD) (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 pre-transplant cytoreduction while not interfering with potential stem cell yield. A stem cell yield supporting two SCTs ($8-10 \times 10^6$ CD34+ cells/kg) is the collection goal recommended in 2009 by the International Myeloma Working Group (IMWG) (26). Identifying induction therapies that are both effective and capable of achieving this goal are paramount; induction and mobilization therapy prior to HDT-SCT should therefore aim to maximize both cytoreduction and stem cell yields.

We assessed both the efficacy and 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 MM patients 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 performed on CD34+ cells from MM patients in an attempt to understand the molecular effects of bortezomib on stem cell mobilization.

MATERIALS AND METHODS

Patients

All patients aged ≥ 18 years with active MM (> 1.0 g/dL serum M-protein, > 0.1 g/dL serum free light chains, > 0.2 g/24-hour urinary M-protein excretion, and/or measurable plasmacytomas, with evidence of MM-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 three successive monthly assessments) to continued initial treatment. Patients were also eligible if they had relapsed (determined in accordance with IMWG criteria (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/mm³; platelet count $\geq 75,000$ /mm³; 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 2 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 two cycles or $<CR$ after four 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 μ g/kg/day 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 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 (AEs) 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 and distributions were estimated using Kaplan-Meier methodology.

Stem cell collection and hematopoietic recovery—Post-induction stem cell collection was initiated when patients' ANC reached $\geq 1.0 \times 10^9$ /L. Leukapheresis was performed using indwelling venous catheters and the COBE Spectra continuous flow cell

separator (Gambro BCT, Lakewood, CO, USA) in the automatic mode for mononuclear cell collection. Large volume (20–24 L) leukapheresis was performed to reach a target of 10×10^6 CD34+ cells/kg. Unmanipulated cells were characterized for nucleated cell counts and CD34, then cryopreserved at 7.5% in dimethylsulfoxide and stored at -190°C . The time to post-transplant ANC recovery ($>0.5 \times 10^9/\text{L}$) and platelet recovery ($>20 \times 10^9/\text{L}$) were recorded.

Gene expression/signaling pathway/plasma analyses—Analyses 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 $10 \mu\text{g/kg/day} \pm$ high-dose cyclophosphamide (3 g/m^2) 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 one month after completing induction.

The gene expression profiles of CD34+ stem cells enriched from leukapheresis product were compared between six and four 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 performing a principal component analysis, leaving five and three samples, respectively. Levels of CXCL12 (SDF-1 α) were compared in six and seven patients, respectively, and angiopoietin-1 levels in plasma were compared between samples from seven and nine 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, Vancouver, BC). The purity of the CD34+ isolate was assessed by flow cytometry using an anti-human CD34-PE antibody (BD Biosciences, San Jose, CA). Total RNA was isolated from the CD34+ cells using the RNeasy Mini kit from Qiagen (Valencia, CA). RNA yield and quality was assayed using a Nanodrop (Thermo Scientific, Wilmington, DE) and the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). 25–200 ng of total RNA were reverse transcribed and amplified using the WT-Ovation Pico RNA Amplification System from NuGEN Technologies (San Carlos, CA). Amplified cDNA (5 μg) was fragmented, labeled with biotin, and hybridized to Affymetrix HG-U133 plus two microarray chips (Agilent, Santa Clara, CA), which contained $>47,000$ transcripts, 38,500 well-characterized human genes, and more than 54,000 probe sets. Raw intensity (.cel) files were imported and pre-processed using the RMA algorithm (29).

Chip data were imported into the GeneSpring GX 115 program (Agilent Technologies, Foster City, CA). 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 performed 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 Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

Quantitative real-time polymerase chain reaction (QPCR)—Total RNA (50 µg) from CD34+ cells was reverse transcribed into cDNA using a qScript cDNA supermix reagent from Quanta Biosciences, Inc. (Gaithersburg, MD) 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 (Bedford, MA). The following TaqMan gene expression assays (ABI Life Technologies, Carlsbad, CA) were used to perform 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 ANKRD36B (Hs00743856_s1). Real-time QPCR was performed in triplicate using the ABI 7900HT machine (Applied Biosystems, Bedford, MA). Comparative delta Ct analysis was performed 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 (IL8) 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 1,500g 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, Minneapolis, MN) according to the manufacturer's protocol, and were based on the average of duplicate samples. 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 two tailed t-test was performed to test the significance of the difference in CXCL12 plasma levels and a Mann-Whitney U t-test was performed 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 performed 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 performed due to the limited number of PFS events in the analysis. All p-values are two-sided with statistical significance evaluated at the 0.05 alpha level. Ninety-five percent confidence intervals (95% CI) were calculated to assess the precision of the obtained estimates. All analyses were performed in SAS Version 9.3 (SAS Institute Inc., Cary, NC) and STATA Version 12.0 (StataCorp, College Station, TX).

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 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). Over 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 six 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); additionally, 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; additionally, there was one VGPR-to-sCR transition and three CR-to-sCR transitions after mobilization. Ten patients underwent stem cell mobilization with non-bortezomib-based regimens; six patients were mobilized with filgrastim alone, and four were mobilized with cyclophosphamide + filgrastim. Of these 10 patients, one patient each achieved CR and VGPR, and two patients achieved PR post-mobilization (Supplementary Table S1). One patient was not mobilized during the study due to septic arthritis.

Stem cell collection and SCT

CD34+ stem cell yields greatly exceeded the study goal of $10 \times 10^6/\text{kg}$ in 23 of 27 (85%) patients who received bortezomib-based mobilization, with a median yield of 23.2×10^6 cells/kg (range $6.8\text{--}294.2 \times 10^6$) obtained within a median of 1 collection day (range 1–5). 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 $4.79\text{--}15.77 \times 10^6$). 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 200mg/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 days and 15 days, respectively. Of the 10 patients who underwent stem cell mobilization with non-bortezomib-containing regimens, eight subsequently underwent SCT and two 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 non-hematologic AEs during induction were hand-foot syndrome (11%) and PN (8%). Diarrhea was the only grade 3 non-hematologic AE reported during mobilization in 1 (4%) patient. In total, 28 (74%) patients experienced PN during DoVeD induction, including nine (24%) with grade 2 PN and three (8%) with grade 3 PN. During bortezomib-based mobilization, six (22%) patients experienced PN, but no grade 3 PN was observed. No additional grade 3 PN events were observed following induction. No serious AEs (SAEs) were reported during induction therapy.

Outcomes

Seven of the 38 (18%) patients had died by the final data cut-off (15 April 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 reached; the 5-year OS rate was 76.4% (95% CI: 56.2%, 88.2%) (Supplementary Fig. S2B).

The effect of multiple patient characteristics (including myeloma type, β_2 -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. Univariate analysis of the factors potentially affecting OS, and multivariate analyses of PFS and OS, could not be performed due to 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 received non-bortezomib mobilization. In total, 12,727 genes were differentially expressed ($p < 0.05$) between the two patient groups (data not shown). After filtering using a cut-off of $p < 0.0025$, 997 genes were determined to be analysis-ready and used for Ingenuity Pathway Analysis. The ten most significantly up- or down-regulated 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 Ingenuity Pathway Analysis. 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; eight of nine genes tested were in agreement with respect to up- or down-regulation. The level of gene expression changes (fold change up or down) varied between the QPCR and microarray datasets (Supplementary Table S3).

IL8 was the highest differentially expressed gene between CD34+ cells from patients receiving bortezomib- and non-bortezomib-containing mobilization (Table 3). In addition, IL8 was identified in a biomarker analysis of the gene expression data using Ingenuity Pathway Analysis with a filter for molecules found in plasma and/or serum (data not shown); we therefore investigated plasma levels of IL8 in two groups. However, there were no significant differences in IL8 plasma levels ($p=0.6057$, data not shown) between the two groups.

Fig. 1 summarizes the five highest-ranked canonical pathways exhibiting significant changes in gene expression between bortezomib- and non-bortezomib-containing mobilization. The highest-ranked canonical pathways (determined using Ingenuity Pathway Analysis 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). In order to determine if 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 determined to be differentially regulated between patients receiving bortezomib- and non-bortezomib-containing mobilization. These genes, which are summarized in Table 4, are also associated with the five highest-ranked canonical pathways.

Due to the known involvement of CXCR4 and its ligand CXCL12 (SDF-1 α) in the homing and migration of multiple stem cell types (32), and due to its prominent role in the ephrin

signalling pathway with known functional activity in migration and chemoattraction (Fig. 2A), we looked at plasma levels of CXCL12 in patients receiving bortezomib- and non-bortezomib-containing mobilization. While gene expression of the CXCR4 receptor in CD34+ cells was upregulated ($p<0.01$, fold change =4.74), plasma levels of CXCL12 were significantly lower in patients receiving bortezomib- ($n=6$) versus non-bortezomib-based ($n=7$) mobilization (mean 1319 pg/mL vs. 2225 pg/mL, $p<0.001$, Fig. 2B).

We also investigated the levels of angiopoietin-1 protein in plasma as ANGPT1 expression in CD34+ cells was also significantly higher in patients receiving bortezomib- ($n=7$) versus non-bortezomib-based ($n=9$) mobilization. Angiopoietin-1 was also identified in a biomarker analysis of the gene expression data using Ingenuity Pathway Analysis with a filter for molecules found in plasma and/or serum, and in the ephrin signalling canonical pathway with known functional activity in cell proliferation. Similar to the CXCL12 results, angiopoietin-1 level was significantly lower in the plasma of patients receiving bortezomib- versus non-bortezomib-containing mobilization (mean 4296 pg/mL vs. 11,969 pg/mL, $p<0.001$, Fig. 2C), suggesting that lower levels of plasma angiopoietin-1 and CXCL12 could potentially be useful as biomarkers of improved CD34+ mobilization in bortezomib-treated patients.

DISCUSSION

This phase 2 study evaluated the efficacy and safety of bortezomib-based secondary induction and mobilization in a cohort of transplant-eligible MM patients who had plateaued after achieving <VGPR with, or having relapsed following, one previous primary induction therapy, which was mostly IMiD-based (and predominantly lenalidomide-based). DoVeD induction showed substantial activity in this patient population, and both the induction and mobilization regimens were well tolerated, with the observed toxicities being consistent with those previously reported for the individual drugs involved (14,33).

DoVeD was shown to be successful for breaking plateaued responses to primary induction therapy, with an ORR relative to pre-DoVeD assessment of 61%, including 18% CR and 39% VGPR. Twenty-seven patients in the study had plateaued at PR with their first induction; DoVeD proved to be a useful regimen for further enhancing response to VGPR or better, thereby potentially providing patients with the associated prognostic benefit associated with this level of response. Notably, as evidenced by the response rates to DoVeD, addition of liposomal doxorubicin in patients with a suboptimal initial response to bortezomib-dexamethasone appeared effective in improving their responses.

The ORR observed with DoVeD secondary induction in this small patient population is notable in the context of previous phase 2 and 3 studies of this combination in previously untreated MM patients, which reported ORR rates of 78–95%, including 42–62% VGPR (4–6,34–36). Our results indicate that substantial activity with DoVeD-like regimens in previously untreated MM 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 MM (19–21), this study is unique in demonstrating activity in patients who received bortezomib-based secondary

induction after achieving maximum incomplete responses to primary induction with IMiD-based regimens.

Previous studies have demonstrated an association between the achievement of VGPR prior to 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 5-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 mobilization with bortezomib + cyclophosphamide + filgrastim, with 10/27 (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 demonstrating the cytoreductive effect of bortezomib in combination with cyclophosphamide (14,15,23), and highlight the utility 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 and Ingenuity Pathway Analysis 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 to microarray data but a variation in the level of fold change between the two methods, as also reported in other studies (37,38). Such inconsistencies are likely due to inherent differences between the two methods, differences in normalization methods, and the limited amount of RNA available for QPCR (n=3–4).

The up-regulation 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 demonstrated 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 vs. 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 HIF1- 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 two 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 co-cultures 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 receptor-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's lymphoma (NHL) demonstrating 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 demonstrated the ability of bortezomib to decrease angiopoietin expression in *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 PI3K, 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 MM 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 prior to transplant (4). Notably, the addition of bortezomib to mobilization or conditioning regimens to further enhance cytoreduction prior to SCT could be more broadly applicable; this agent is being investigated as a component of both, supported by preclinical data demonstrating 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 ligands as potentially of importance in CD34+ stem cell migration from the bone marrow, a finding that warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

This study demonstrated substantial activity with sequential use of non-cross-resistant agents/regimens as induction in myeloma, with secondary induction with DoVeD (bortezomib-dexamethasone $\pm$ liposomal doxorubicin) resulting in further tumor burden reduction in patients following stalled or plateaued responses to primarily immunomodulatory-drug-based initial induction. Furthermore, the approach of adding bortezomib to standard stem cell mobilization therapy (cyclophosphamide and filgrastim) resulted in deeper responses, providing further evidence for the cytoreductive effect of bortezomib in combination with cyclophosphamide. 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.

Fig 1.

Top five 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 $-\log(p\text{-value})$ which is calculated in IPA using a right-tailed Fisher's 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.

A

B

C

Fig 2.

(A) Overlay of significant differential gene expression data ($p < 0.05$, fold change = 2.0) onto the canonical ephrin signaling pathway (Ingenuity pathway analysis). CXCR4 and angiopoietin-1 play prominent roles in migration and proliferation, respectively, as highlighted with blue arrows. Green and red highlighting indicate genes significantly upregulated and down-regulated, respectively. Plasma levels of (B) CXCL12 and (C)

angiopoietin-1 were significantly lower ($p<0.001$) in patients received bortezomib- versus non-bortezomib-containing mobilization.

Table 1

Patient baseline demographics and disease characteristics at initiation of DoVeD induction.

Characteristic*	N=38
Median age, years (range)	61 (27–76)
Age <70 years, n (%)	34 (89)
Age ≥70 years, n (%)	4 (11)
Male, n (%)	21 (55)
Median β_2 -microglobulin, mg/L (range)	2.05 (1.0–10.2)
Median albumin, g/dL (range)	3.5 (2.0–4.3)
Durie–Salmon Stage, n (%)	
Ia*	1 (3)
Ila	19 (50)
IIIa	17 (45)
IIIb	1 (3)
ISS Stage, n (%)	
I	18 (47)
II	17 (45)
III	3 (8)
Abnormalities by FISH, n (%)	
Trisomy 11	10 (26)
Hyperdiploidy	7 (18)
t(4;14)	4 (11)
p53	4 (11)
del(17p)	1 (3)
t(11;14)	5 (13)
t(14;16)	2 (5)
None	16 (42)
Prior induction therapy	
Lenalidomide + dexamethasone ± clarithromycin	21 (55)
Thalidomide + lenalidomide + dexamethasone ± clarithromycin	8 (21)
Thalidomide + dexamethasone	3 (8)
Thalidomide only	2 (5)
Pulsed dexamethasone only	2 (5)
Melphalan + cyclophosphamide	1 (3)
Dexamethasone followed by single-agent lenalidomide	1 (3)
Best response to prior induction therapy	
CR [†]	1 (3)
PR	27 (71)
Stable disease	10 (26)

Abbreviations: CR, complete response; FISH, fluorescence in situ hybridization; ISS, International Staging System; PR, partial response.

* As assessed by the treating physician, this patient had active myeloma based on the IMWG criteria.

[†]CR with melphalan + cyclophosphamide (but with progression of disease after induction).

Table 2

CD34+ stem cell collection and hematologic recovery post-SCT, by patient, in patients receiving bortezomib-based mobilization.

Patient	Number of collection days	Days from start of mobilization to start of collection	Total CD34+ stem cells collected ($\times 10^6/\text{kg}$)	Total CD34+ stem cells infused ($\times 10^6/\text{kg}$)	Days to ANC recovery ($>1.5 \times 10^9/\text{L}$)	Days to platelet recovery ($>20 \times 10^9/\text{L}$)
1	1	18	21.2	5.78	14	20
2	1	18	47.4	13.22	11	13
3	1	19	22	9.87	13	22
4	1	18	17.9	9.03	10	15
5	4	19	40.6*	5.44	11	21
6	1	18	19.9	9.24	10	16
7	3	19	294.2**	17.73	10	17
8	2	17	13.8	6.32	13	24
9	5	18	9.25	4.25, 2.74	11	18
10	2	17	21.4	9.05	16	21
11	1	24	50.0	no SCT†	16	16
12	2	19	66.1	12.83	11	11
13	1	18	30.4	7.38	11	11
14	2	16	43.6	10.02	12	15
15	1	19	51.0	12.72	13	11
16	1	17	15.6	5.31	11	13
17	1	17	6.8	6.66	12	13
18	1	17	31.7	9.20	11	20
19	1	17	7.8	7.40	10	11
20	1	16	43.9	11.06	10	21
21	1	18	23.2	no SCT†	10	11
22	1	17	14.0	3.47	16	16
23	2	17	13.4	3.96	12	11
24	1	16	32.8	7.92	18	14
25	1	20	11.5	5.63	11	18

Patient	Number of collection days	Days from start of mobilization to start of collection	Total CD34+ stem cells collected ($\times 10^6/\text{kg}$)	Total CD34+ stem cells infused ($\times 10^6/\text{kg}$)	Days to ANC recovery ($>1.5 \times 10^9/\text{L}$)	Days to platelet recovery ($>20 \times 10^9/\text{L}$)
26	1	15	29.4	8.2	17	22
27	3	18	27.2	10.2	10	14
Median (range)	1 (1–5)	18 (15–24)	23.2 (6.8–294.2)	8.2 (3.4–17.7)	11 (10–18)	16 (11–24)

Abbreviations: ANC, absolute neutrophil count; SCT, stem cell transplantation

* 20.4×10^6 cells collected after 2 days

** 86.3×10^6 cells collected after 1 day

[†] Patient opted to defer HDT-SCT until later.

Table 3

The ten most significantly up- or down-regulated genes (p 0.0025, fold change [FC] in expression >2.0) identified in gene expression analysis of CD34+ cells from patients receiving bortezomib- versus non-bortezomib-based mobilization.

Top upregulated	FC up	Function/Role_up	Top down-regulated	FC down	Function/Role_down
IL8/Interleukin 8	23.72	Chemokine, chemotaxis, migration	NUMA1/nuclear mitotic apparatus protein	-12.06	Microtubule binding, mitosis
RAC1/ras related C3 botulinum toxin substrate 1	19.45	GTP-ase, member, protein binding, migration, invasion	MUC6/mucin 6	-11.3	Extracellular matrix constituent, survival, protein binding
KLF10/Kruppel-like factor 10	18.44	DNA binding, transcription factor, proliferation, polyubiquitination	CPT1B/carnitine palmitoyltransferase 1B	-10.39	Cell death, protein binding, transferase activity
VBPI/von Hippel-Lindau binding protein	17.82	Unfolded protein binding	NCOR2/nuclear receptor corepressor 2	-6.72	DNA and protein binding, receptor activity, growth, motility, self renewal, proliferation
TNFSF4/ tumor necrosis factor 4, apoptosis, proliferation, recruitment	17.2	Cytokine activity, TNF receptor binding, apoptosis, proliferation, recruitment	MUC4/Mucin 4	-6.35	ERBB2 receptor binding, Extracellular matrix, apoptosis, motility, colony formation
PPT1/palmitoyl-protein thioesterase 1	16.98	Hydrolase activity, apoptosis, development	MS12/musashi homologue	-5.91	Nucleotide and RNA binding, development, proliferation
AP1S2/Adapter Protein 1	16.53	Protein transporter activity, cell cycle, differentiation	INTS3/integrator complex, subunit 3	-5.66	Protein binding, DNA damage, cell death, ser/thr phosphorylation
CERS6/Ceramide synthase 6	15.73	Sequence specific DNA binding/transcription factor activity, permeabilization	ABCA2/ATP binding cassette	-5.52	ATPase activity, transmembrane movement, ATP binding, nucleotide binding
UBE2A/ubiquitin conjugating enzyme 2	15.36	Ubiquitin protein ligase activity, proliferation, cell death	ANKRD36B/ankyrin repeat domain 36B	-5.21	Ion channel Inhibitor
GLUD1/Glutamate dehydrogenase	14.78	Protein binding, cell survival, apoptosis	KCNIP2/Kv channel interacting protein	-5.08	Potassium ion channel activity, Calcium binding, ER

Table 4

Differential gene expression in CD34+ cells in the ephrin signaling pathway between patients receiving bortezomib- and non-bortezomib-based mobilization.

Symbol	Entrez gene name	p-value	Fold change	Location	Function	Movement/migration
ABI1	abl-interactor 1	6.14E-04	7.903	Cytoplasm	Nucleotide binding	Subcellular movement
ACTR2	ARP2 actin-related protein 2 homolog (yeast)	2.33E-03	8.778	Plasma membrane	Actin/nucleotide binding	Subcellular movement
ARPC3	actin related protein 2/3 complex, subunit 3, 21kDa	2.35E-03	4.622	Cytoplasm	Protein binding	Increased migration of NIH3T3 and HEK293
CFL2	cofilin 2 (muscle)	2.33E-03	6.201	Nucleus	Protein binding	Increased migration of vascular smooth muscle cells
GNAQ	guanine nucleotide binding protein (G protein), q polypeptide	2.29E-03	6.116	Plasma membrane	Enzyme	Migration of HUVEC
GNAS	GNAS complex locus	1.86E-03	7.411	Plasma membrane	Enzyme	Migration of prostate cells
GNB1	guanine nucleotide binding protein (G protein), beta 1	1.11E-03	4.562	Plasma membrane	Enzyme	Migration of smooth muscle cells, jurkat cells and huvecs, SDF1
GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	1.65E-03	-2.799	Plasma membrane	Ion channel	Migration of neurons
GRIN2C	glutamate receptor, ionotropic, N-methyl D-aspartate 2C	1.46E-03	-3.081	Plasma membrane	Ion channel	Projection neurons
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.56E-03	9.561	Plasma membrane	Transmembrane receptor	Migration in cell lines includes B lymphocytes
MAP2K1	mitogen-activated protein kinase kinase 1	1.08E-03	5.886	Cytoplasm	Kinase	Migration in cell lines including B lymphocytes
MAPK1	mitogen-activated protein kinase 1	1.59E-03	6.554	Cytoplasm	Kinase	Migration in cell lines including bone marrow cells
NCK2	NCK adaptor protein 2	2.24E-03	4.436	Cytoplasm	Kinase	None
PGF	placental growth factor	1.62E-03	-2.301	Extracellular space	Growth factor	Decreased expression causes increased migration in cells including progenitor cells
RAC1	ras-related C3 botulinum toxin substrate 1	1.22E-03	19.450	Plasma membrane	Enzyme	Migration in cell lines
RAP1A	RAP1A, member of RAS oncogene family	1.86E-03	5.896	Cytoplasm	Enzyme	Migration in cell lines
RAP1B	RAP1B, member of RAS oncogene family	1.25E-03	3.227	Cytoplasm	Enzyme	Migration in cell lines
SOS2	son of sevenless homolog 2 (Drosophila)	1.61E-03	4.056	Cytoplasm	Protein binding/catalytic domain	None
WIPF1	WAS/WASL interacting protein family, member 1	1.81E-03	6.030	Cytoplasm	Actin/protein binding	None