DIFFERENTIAL EFFECTS OF PROTEASOME INHIBITION BY BORTEZOMIB ON MURINE ACUTE GRAFT-VERSUS-HOST DISEASE (GVHD): DELAYED ADMINISTRATION OF BORTEZOMIB RESULTS IN INCREASED GVHD-DEPENDENT GASTROINTESTINAL TOXICITY

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ABSTRACT

We have recently demonstrated that the proteasome inhibitor, bortezomib, administered immediately following murine allogeneic bone marrow transplantation (BMT) resulted in marked inhibition of acute graft-versus-host disease (GVHD) with retention of graft-versus-tumor effects. We now assessed the effects of delayed bortezomib administration (5 or more days post-BMT) on GVHD. Recipient C57BL/6 (H2b) mice were lethally irradiated and transplanted with bone marrow cells and splenocytes from MHC-disparate BALB/c (H2d) donors. In marked contrast to the effects of bortezomib on GVHD prevention when administered immediately after BMT, delayed bortezomib administration resulted in significant acceleration of GVHD-dependent morbidity. No toxicity was observed following delayed bortezomib administration in models where donor T cells were not co-administered, indicating that these deleterious effects were critically dependent on GVHD induction. The increase in GVHD susceptibility even occurred when late administration of bortezomib was preceded by early administration. Pathological assessment revealed that significant increases in gastrointestinal lesions following delayed bortezomib administration during GVHD. This pathology correlated with significant increases of type I TNF-α receptor transcription in gastrointestinal cells and with significant increases of TNF-α, IL-1β and IL-6 levels in the serum. These results indicate that the differential effects of proteasome inhibition with bortezomib on GVHD are critically dependent on the timing of bortezomib administration.
INTRODUCTION

The occurrence of acute graft-versus-host disease (GVHD) remains one of the most significant causes of morbidity following allogeneic bone marrow transplantation (BMT). GVHD is caused by administration of donor T cells into a genetically-disparate recipient. The pathophysiology of GVHD is a complex process that can be conceptualized in three phases. In the first phase, cytoreductive conditioning regimen causes immunosuppression of the recipients and damage to host tissues, including a self-limited burst of inflammatory cytokines. In the second phase, donor T cells recognize alloantigens on host APCs and these activated T cells then proliferate and differentiate into effector cells. The second phase is critical for the amplification of the systemic inflammatory response, in which donor T cells also contribute to the inflammatory cytokine network. In the third phase, target tissues undergo apoptosis mediated by cellular effectors and inflammatory cytokines such as TNF-α and interferon-γ, and further host tissue injury establishes a positive inflammatory feedback loop. Solid organs attacked during acute GVHD include the gut, liver, lungs and skin.

The proteasome is a multicatalytic proteinase complex responsible for the degradation of most intracellular proteins, including proteins crucial to cell cycle regulation and apoptosis. Bortezomib (Velcade™, formerly PS-341) is the first of its class of proteasome inhibitors to be tested in humans and has shown promising activity in several tumor types, especially in hematologic malignancies. It has recently been approved as a therapy in multiple myeloma. Bortezomib has been demonstrated to exert numerous biological effects that include blocking the activation of the transcription factor NF-κB. NF-κB is implicated in the regulation of many genes that code for mediators of the immune and inflammatory responses.
We have recently demonstrated that proteasome inhibition using bortezomib can markedly inhibit the generation of acute GVHD in mice after allogeneic BMT. Importantly, significant anti-tumor effects were maintained suggesting that bortezomib may be of use to improve the efficacy of BMT. In that study, we found that bortezomib administered immediately after BMT reduced donor-derived T cell expansion by inhibiting cell proliferation and selectively inducing apoptosis in the activated donor alloreactive T cell population. Another mechanism by which bortezomib can limit GVHD may involve the blockade of NF-κB activity resulting in a decrease in inflammatory cytokines produced by donor T cells and damaged host tissues after cytoreductive conditioning. However, NF-κB has been shown to have both pro-apoptotic and anti-apoptotic functions and can affect both pro- and anti-inflammatory responses. It is possible that GVHD progression could also be enhanced by blocking NF-κB activity and thereby augmenting TNF-mediated cellular injury. Therefore, it was important to investigate the effects of bortezomib administration during ongoing GVHD.

We report here that the differential effects of proteasome inhibition with bortezomib on a murine GVHD model is determined by the timing of bortezomib administration. Delayed bortezomib administration resulted in marked acceleration and manifestation of GVHD-dependent pathology, particularly in the gut following BMT. This was correlated with increased transcription of type I TNF-α receptor in the gut and the presence of pro-inflammatory cytokines in the serum. These results suggest that extreme caution must be used with delayed administration of bortezomib in combination with T cell replete allogeneic BMT.
MATERIALS AND METHODS

Animals. Female BALB/c (H2^d), C57BL/6 (B6, H2^b) and immunodeficient C.B-17^{scid/scid} (SCID, H2^d) mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were kept in specific pathogen-free conditions. All animal protocols were approved and in vivo studies were performed at each of the two animal facilities (the National Cancer Institute at Frederick and the University of Nevada, Reno). Mice were between 8 and 12 weeks of age at the start of the experiments.

Reagent. The proteasome inhibitor, bortezomib, was kindly provided by Millennium Pharmaceuticals (Cambridge, MA). Stock bortezomib solution (1 mg/mL) was prepared in Dulbecco's phosphate-buffered saline solution (PBS) and stored at −70°C for up to 2 months prior to use. Bortezomib solutions were protected from light at all times. The stock solutions were thawed and diluted to 0.075 mg/mL in PBS immediately prior to use.

Cell preparation. Bone marrow cells (BMCs) suspensions were prepared by gently releasing cells from the backbones, femurs, and tibiae into PBS with a mortar and pestle, filtering through a mesh filter to remove particulates, and washing the cell suspensions twice. Spleen cell preparations were prepared by gently crushing the tissues to release the cells. Preparations were filtered to remove debris and washed twice in PBS for injection. Cell counts were performed on a Coulter Z1 cell counter (Coulter Electronics, Hialeah, FL, USA).

In Vivo Studies. Induction of GVHD studies were performed at each of the two animal facilities (the National Cancer Institute at Frederick and the University of Nevada, Reno). C57BL/6 (B6, H2^b) or CB-17 SCID (H2^d) mice were used as recipients in the two GVHD model systems separately. B6 recipient mice received myeloablative doses (900–950 cGy) of total body
irradiation (TBI) from a $^{137}$Cesium source. Irradiation was followed by the infusion of 1.0 or 1.5 x $10^7$ BALB/c BMCs i.v. with or without BALB/c splenocytes (SCs; 5–20 x $10^6$ cells i.v.) as a source of allogeneic T cells. CB-17 SCID recipient mice did not receive conditioning and were transplanted with B6 splenocytes (SCs; 40 x $10^6$ cells i.v.) as a source of allogeneic T cells. Recipient B6 or CB-17 SCID mice then received PBS, or bortezomib in PBS at a dose of 15 µg (i.v. or i.p., with comparable results) at the indicated time phase post-cell infusion. Mice were monitored and weighed weekly. All moribund mice were euthanized. All experiments were performed at least three times with 5–10 mice per group.

**Histology.** Tissues (liver, small and large intestine) from the mice were placed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were evaluated and graded in coded fashion by a veterinary pathologist (MRA) as previously described$^{17}$. A semi-quantitative scale from 0 to 4 was used where histopathological changes were identified as minimal = 1, mild = 2, moderate = 3, severe = 4. Cumulative histopathology scores were calculated based on the sum of individual changes of 3-4 parameters in each organ (villous blunting, crypt cell hyperplasia, crypt cell apoptosis and inflammation in the small intestine; goblet cell depletion, sloughing of epithelial cells into the lumen, and crypt cell apoptosis in the colon; and vacuolation, necrosis and oval cell hyperplasia in the liver). Each experiment consisted of 3 to 6 mice per group and 4 independent experiments were assessed. Images were visualized using an Olympus Vanox AHBS3 microscope with an Olympus SPlan Apo 20 x objective. A Diagnostic Instrument Spot RT color digital camera utilizing Spot software version 4.0.2 was used to acquire the images.

**RNAsen Protection Assay (RPA).** Total small intestine and liver RNA was isolated using RNA STAT-60 (Tel-test, Friendswood, TX) according to manufacturer’s instructions. mRNA
expression was then examined using the BD Riboquant RNase Protection Assay System (mouse APO-3 probe set, PharMingen, San Diego, CA) as directed. Briefly, $^{32}$P-UTP labeled antisense RNA probes were synthesized and then hybridized with total small intestine and liver RNA overnight (16 hrs). The following day, free RNA stands and excess probe were digested with RNase A and T1, then precipitated, resuspended in 5ul of loading buffer, and run on a 4.75% polyacrylamide gel. The gel was then absorbed onto gel blot paper and dried on a Slab Gel Dryer at 80° C (Savant, Holbrook, New York), followed by overnight exposure and autoradiography using the Bio-Rad GS-525 Molecular Imager System (Hercules, CA). Band densities were determined using Molecular Analyst Software (Bio-Rad) and normalized against the L32 and GAPDH probes for each sample. Samples for RNAse Protection Assay from 2 (liver) and 4 (gut) independent experiments consisted of 3 – 5 samples per group in each independent experiment.

**Cytokine analysis.** Serum was collected on day 13 post-BMT. Serum cytokine levels were determined by multiplex analysis on a Luminex (Austin, TX), using mouse cytokine-specific bead sets and standards according to the manufacturer’s instructions (R & D Systems, Minneapolis, MN). Serum samples from three independent experiments with 3 – 5 serum samples per group in each independent experiment.

**Statistics.** Survival data were plotted by the Kaplan–Meier method and analyzed by the log-rank test. Serum and RPA data were analyzed by the unpaired Student $t$ test with Welch’s correction (two-tailed P value). Pathology scores were evaluated with the Mann-Whitney Test. A $P$ value of <0.05 was considered significant.
RESULTS

Delayed administration of bortezomib results in accelerated GVHD morbidity following allogeneic BMT. We have previously observed that bortezomib, administered immediately after allogeneic BMT, resulted in increased survival and protection from GVHD. Therefore, we next assessed the effects of bortezomib given at later time-points when GVHD was ongoing. Using a fully MHC-mismatched BMT model [BALB/c (H2^d) bone marrow cells and splenocytes into lethally irradiated C57BL/6 (H2^b) recipients], we found that early administration of bortezomib (day 0-2 post-BMT) consistently resulted in significant protection from GVHD (Figure 1A, B). In marked contrast, when bortezomib was administered at later time points (day 5-7 post-BMT) the mice rapidly succumbed to acute GVHD mortality, sometimes showing overt acute GVHD within 12 hours of the first injection and all succumbing within several days from the first day of injection (Figure 1A). Use of donor purified T cells resulted in findings that were comparable with the use of whole splenocytes containing the equivalent dose of T cells (data not shown).

When bortezomib was administered early to prevent GVHD and then continued with the delayed administration, significant increases in GVHD morbidity occurred (Figure 1B), suggesting the augmentation of GVHD associated with delayed bortezomib administration was dominant over the protection seen with early administration.

It has been shown that myeloablative conditioning with TBI for BMT can result in gastrointestinal damage and inflammatory cytokines production. This could be a contributing factor with the increased gut toxicity observed following delayed bortezomib administration. To exclude the influence of cytoreductive conditioning with TBI, we next performed GVHD studies using B6 (H2^b) splenocytes injected into non-irradiated...
immunodeficient C.B-17 SCID (H2<sup>d</sup>) mice (Figure 1C). Administration of bortezomib on the day of cell transfer protected the mice from lethal GVHD while mice that received delayed bortezomib rapidly succumbed to GVHD. In addition, no morbidity was observed in mice that received bortezomib without adoptive transfer of allogeneic spleen cells (SCID recipients, data not shown; allogeneic BMT, Figure 2A, B). The results demonstrated that the increased GVHD-dependent morbidity following delayed bortezomib administration was not dependent on conditioning of the recipients by irradiation as the CB-17 SCID recipients also succumbed earlier to GVHD.

*Delayed administration of bortezomib results in the appearance of GVHD morbidity using doses of donor splenocytes alone that produce no overt pathology.* As the delayed administration of bortezomib results in a rapid induction of GVHD mortality, we next performed dose responses of donor splenocytes to ascertain the extent of GVHD augmentation with delayed bortezomib administration. The results demonstrate that even at low doses of splenocytes, in which no outward signs of GVHD were apparent, the delayed administration of bortezomib resulted in significantly ($P < 0.005$) increased GVHD morbidity (Figure 2 C-E). Thus, delayed bortezomib administration can result in the occurrence of acute GVHD in instances where no evidence of overt GVHD would normally be detected.

Pathologic evaluation indicated that recipients of splenocytes and delayed bortezomib treatment had marked increases in gut lesions in the small intestine and colon (Figures 3, 4) compared to control recipients. The small intestine had greater villous blunting and fusion, ulceration and inflammation. Crypt cell apoptosis and hyperplasia were also more extensive than in the recipients receiving splenocytes alone. Sections of colon tissue showed evidence of goblet cell depletion with sloughed cells in crypt lumens, multifocal ulceration and inflammation. As in the
small intestine, crypt cell hyperplasia was more extensive than in the recipients receiving
splenocytes alone. The presence of microscopic lesions was contingent on the administration of
both splenocytes and delayed bortezomib administration as no pathological damage or GVHD
mortality was observed when bortezomib was administered at this or any other time point
without splenocytes (Figure 2A, B, Figure 3). In the liver, there were no significant differences
in the lesions present between the two treatment groups that received splenocytes (Figure 3).
This suggests that the gut is the primary target for this increased mortality after delayed
bortezomib administration following allogeneic BMT. Thus, the data indicate that the timing of
bortezomib administration has a pivotal role in protection or increased GVHD pathology after
BMT and the gut is the primary GVHD target organ affected.

*Delayed administration of bortezomib is associated with increased TNFR1 mRNA levels in the
gut of GVHD mice.* Because histopathological examination had revealed that the gut was
particularly affected with delayed bortezomib administration, we analyzed the tissue for
expression of genes associated with killing pathways. At 6 hours after delayed (day 12-13 post-
BMT) bortezomib administration, the small intestine and liver were assessed by RPA for mRNA
levels of *Fas, FasL,* and *TNFR1* (p55). The results demonstrate that significant increases in
*TNFR1* (type I TNF-α receptor), the principal pro-inflammatory and pro-apoptotic receptor for
TNF-α,²⁰ were observed in the small intestine, but not the liver, of mice receiving splenocytes
and delayed bortezomib treatment compared to recipients receiving either delayed bortezomib
treatment (data not shown) or splenocytes alone (Figure 5 A-C). No significant differences in
*Fas* or *FasL* were observed (data not shown) suggesting that the TNF pathway may be
particularly targeted in the gut of recipients receiving splenocytes after delayed bortezomib
administration. Thus, up-regulating type I TNF-α receptor expression in small intestine may be,
in part, the mechanism of increased susceptibility of GVHD mice to immune-mediated target
damage and lethality after delayed bortezomib administration.

*Increases in serum cytokine levels in GVHD mice after delayed bortezomib administration.* We
next assessed the levels of pro-inflammatory cytokines at 6 hours after delayed (day 12-13 post-
BMT) bortezomib administration. Serum levels of the pro-inflammatory cytokines TNF-α, IL-1β
and IL-6 were all significantly (P < 0.05) increased in the recipients receiving splenocytes and
delayed bortezomib treatment compared to recipients receiving splenocytes and vehicle control
(Figure 6). Delayed administration of bortezomib resulted in cytokine increases only in the
recipients with splenocytes but not in the recipients without splenocytes (Figure 6). These results
indicate that despite the ability of proteasome inhibition by bortezomib to block NF-kB,
increased pro-inflammatory cytokines were detected after delayed administration of bortezomib
during ongoing GVHD. Consistent with the histopathology data, increases in TNF-α, IL-1β and
IL-6 levels as well as gut tissue *TNFR1* levels after delayed bortezomib administration were
critically dependent on GVHD induction.
DISCUSSION

The results presented here definitively demonstrate that proteasome inhibition with bortezomib can prevent acute GVHD when administered immediately after BMT but can also promote GVHD-dependent toxicity when administration is delayed. This increase in GVHD mortality was striking in that it could be observed even when lower amounts of donor T cells were administered such that no outward manifestations of GVHD (i.e. diarrhea, posture or grooming changes) was observed in the recipients not receiving delayed bortezomib and when GVHD was prevented by early administration of bortezomib. These results are consistent with our previous study that GVHD prevention by early administration of bortezomib is due to a lowering or dampening of the donor T cell responses. Reduced T cell responses could still be augmented by the later administration of bortezomib. This may also explain how graft-versus-tumor responses were still maintained following early bortezomib treatment.

Bortezomib exerts numerous biological effects that include blocking the activation of the transcription factor NF-κB. NF-κB is implicated in the regulation of many genes that code for mediators of the immune and inflammatory responses. GVHD progression is accompanied by inflammatory cytokine production and inflammatory cytokine-mediated target tissue damage. Therefore, not only immune cells (donor T cells, recipient dendritic cells) but also non-immune cells (recipient target tissues) should be affected by delayed bortezomib administration. NF-κB has been shown to play a protective role in TNF-α-induced cell death. Therefore, blocking NF-κB activity in GVHD target tissues may enhance GVHD damage by enhancing TNF-mediated cellular injury. Our data indicates that the gut is the primary organ made more susceptible to GVHD attack after delayed bortezomib administration. Other studies demonstrate a strong
association with NF-κB and protection from intestinal epithelial apoptosis\textsuperscript{19,21,22} and suppression of anti-apoptotic molecules of the IAP family upregulation by the proteasome inhibitor MG132 with the sensitization of normal enterocytes to Fas-induced apoptosis.\textsuperscript{23} Additionally, it has been demonstrated that NF-κB can protect enterocytes from TNF-mediated death.\textsuperscript{24} Consistent with this observation, it has been demonstrated that NF-κB inhibition could prevent inflammation and yet result in increased cell injury in an intestinal ischemia model.\textsuperscript{25} NF-κB blockade may play a similar role in GVHD by suppressing the initial phase of inflammation and the later phase by increasing the susceptibility of GVHD target organs to immune-mediated apoptosis. Type I TNF-α receptor (\textit{TNFR1}) is the principal pro-inflammatory and pro-apoptotic receptor for TNF-α,\textsuperscript{20,25} and the data presented here demonstrating increased mRNA levels of \textit{TNFR1} would support this pathway as being a potential component of the mechanism(s) by which bortezomib accelerated GVHD-induced gut pathology. It has been demonstrated that \textit{TNFR1}-induced apoptosis involves two sequential signaling complexes. Failure to activate NF-κB (the initial step in the signaling process) results in cell death.\textsuperscript{26} Therefore, delayed bortezomib administration increased susceptibility to GVHD target organ (gut) damage and lethality may involve multiple steps. Donor T cells, as well as cytokines released by donor T cells, are necessary to trigger immune-mediated damage. Delayed bortezomib administration may act, in part, to enhance this damage by upregulating type I TNF-α receptor expression, although bortezomib may induce apoptosis by multiple pathways, some of which may be independent of the NF-κB pathway.\textsuperscript{27} No protection from bortezomib-mediated accelerated morbidity was observed when mice deficient in TNFR1 were used (data not shown). This would suggest that
TNFRII or other mediators may also contribute to the toxicity associated with delayed bortezomib administration.

A previous study has shown that NF-κB activation in leukocytes recruited during the onset of inflammation is associated with pro-inflammatory gene expression, whereas activation during the resolution of inflammation is associated with the expression of anti-inflammatory genes and the induction of apoptosis. Inhibition of NF-κB during the resolution of inflammation protracts the inflammatory response and prevents apoptosis. This suggests that NF-κB has an anti-inflammatory role in vivo involving the regulation of inflammatory resolution. A similar phenomenon may be occurring with donor T cells during GVHD progression in the GVHD models presented here.

Even though previous studies demonstrated that NF-κB activation provides radioprotection to the intestinal epithelium, we have shown that delayed bortezomib administration dependent gut damage in our model is not dependent on radiation damage. The results showing that recipients of grafts with minimal (bone marrow source only) T cells and receiving bortezomib at any time point lack pathological damage or morbidity indicates that bortezomib-related lethality in our model is dependent on GVHD related immune-mediated attack. The demonstration that adoptive allogeneic lymphocyte transfer into immunodeficient SCID mice (without conditioning such as TBI) results in increased GVHD susceptibility after delayed bortezomib administration confirms that bortezomib-related lethality is due solely to GVHD processes.

As GVHD is a complex and multi-stage disease process, it is perhaps not surprising that bortezomib exhibits differential effects contingent on the timing of administration. The induction phase of GVHD appears to be the most susceptible phase for successful intervention. It has been
demonstrated that administration of cytokines such as IL-2, IL-12, administration of cellular therapy such as donor-type NK cells, and the presence of cytokines such as interferon-γ, are all protective only if administered or present early after BMT. If administration or presence of any of the cytokines or immune cells is delayed, accelerated GVHD has been reported. Given that proteasome inhibitors have been demonstrated to sensitize neoplastic cells to chemotherapeutics and immune lytic molecules such as TRAIL or TNF-α, it is also perhaps not surprising that normal cells and tissues may also be sensitized to subsequent immune attack. It remains to be determined if augmented anti-tumor responses can also be observed during this stage and if there is a means to prevent the GVHD-dependent toxicity without loss of GVT.

The results presented here demonstrate the two facets of proteasome inhibition by bortezomib in GVHD, as determined by the timing of bortezomib administration. Our data indicate that bortezomib could be potentially utilized at any time following T cell-depleted allo geneic BMT models. However, extreme caution must be used with delayed administration of bortezomib in combination with T cell replete allo geneic BMT as exacerbation of GVHD-dependent pathology, particularly in the gut, may occur.
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FIGURE LEGENDS

Figure 1. The paradoxical effect of proteasome inhibition with bortezomib on the alteration of GVHD development and mortality as determined by the timing of bortezomib administration. A and B: B6 (H2b) recipients of BALB/c (H2d) 15 million bone marrow and 20 million spleen cells were treated with or without 15 µg per dose of bortezomib daily. Bortezomib protected mice from GVHD mortality when administration was daily from day 0 through +2 post-BMT. Significant increases in survival were observed in early bortezomib-treated mice (●) compared with GVHD control (vehicle control-treated) mice (■) (A & B: *P* < 0.001). Conversely, bortezomib accelerated GVHD mortality when administration was from day +5 through +7 (A) or bortezomib treatment from day 0 through +2 and day+5 through +7 (B) post-BMT. Significant decreases in survival were observed in late bortezomib-treated mice (▲) compared with GVHD control (vehicle control -treated) mice (■) (A: *P* < 0.001) and in continuous bortezomib-treated mice (▲) compared with GVHD control (vehicle control -treated) mice (■) (B: *P* < 0.001). Results from one of three independent experiments are presented for A and B. Each experiment consists of 5–10 mice per treatment group. C: C.B-17 SCID (H2d) recipients of B6 (H2b) 40 million spleen cells were treated with or without 15 µg per dose of bortezomib for one day. Significant decreases in survival were observed in day+5 bortezomib-treated mice (▼) compared with GVHD control (vehicle control -treated) mice (■) (A: *P* < 0.001) and in day+12 bortezomib-treated mice (▲) compared with GVHD control (vehicle control -treated) mice (■) (B: *P* < 0.001). Results from one of three independent experiments are presented. Each experiment consists of 4–10 mice per treatment group.
Figure 2. Dose response of donor spleen cells necessary for the increased GVHD mortality by delayed bortezomib administration. A and B: B6 (H2b) recipients of BALB/c (H2d) 15 million bone marrow cells with or without 15 million spleen cells (SC) were treated with 15 µg per dose of bortezomib daily. Morbidity was not observed in mice that received bone marrow cells but not SC and delayed bortezomib administration from day+5 through +7 (A) or bortezomib treatment from day 0 through +2 & from day+5 through +7 (B) post-BMT. Significant decreases in survival were observed in delayed bortezomib-treated mice with SC (▼, ▲) compared with delayed bortezomib-treated mice without SC mice (●; A, B: \( P < 0.001 \)). C, D and E: B6 (H2b) recipients of BALB/c (H2d) 15 million bone marrow with 15 million (C), 10 million (D) or 5 million (E) spleen cells were treated with or without 15 µg per dose of bortezomib daily. Delayed bortezomib treatment from day+12 through +14 post-BMT accelerates GVHD mortality. Significant decreases in survival were observed in bortezomib-treated mice (▼) compared with mice that received PBS (no bortezomib-treatment) (■; \( P < 0.005 \)). Results from one of three independent experiments are presented. Each experiment consisted of 5–10 mice per treatment group.

Figure 3. Effects of delayed bortezomib administration on pathologic damage to target organs of GVHD. Significant increases in histological changes were observed in the small and large intestine, but not the liver, of mice treated with delayed bortezomib on day +12 or day+12 through day+13 at 15µg/mouse (i.v.). Mice were assessed 16 hours after a single bortezomib or vehicle control (VC) injection (day+12) or 6 hours after 2nd injection (day+13) of bortezomib or VC, respectively. A total of 3 to 6 mice per experimental group in each of four independent experiments were assessed. Tissues were assessed for histological changes in 3 to 4 parameters...
as described in Materials and Methods and the sum of these scores are represented for each tissue. Histological changes for each parameter were assessed and graded where 0 = normal, 1 = minimal; 2 = mild; 3 = moderate; 4 = severe. Comparison of cumulative histopathological scores between the groups with splenocytes were analyzed by Mann-Whitney Test (P < 0.05).

**Figure 4.** Delayed administration of bortezomib significantly increases gut histopathological damage in mice with GVHD. B6 (H2^b^) recipients of BALB/c (H2^d^) 10 million bone marrow and 15 million spleen cells were treated with or without 15 µg per dose of bortezomib daily on day 12 post-BMT. The next day (day 13) mice were euthanized and gut tissue was collected, processed and stained with H & E. A—C, Small intestine: Small intestine from mice without delayed bortezomib treatment (A) have hyperplastic crypts(c) but normal villi (v). In contrast, the small intestines from mice with delayed bortezomib treatment (B, C) have villous blunting and fusion (v), hyperplastic crypts(c) and an inflammatory infiltrate (arrow). Other areas of small intestine are ulcerated (u) with a base of inflammatory cells. D—F, Colon: Colons from mice without delayed bortezomib treatment (D) display relatively normal morphology. In contrast, colons from mice with delayed bortezomib treatment have increased (E) goblet cell depletion and inflammatory cells (arrow) in the lamina propria. Other areas of colon (F) are ulcerated (u), have sloughed cells in crypt lumens (c) and inflammation in the ulcer base and the submucosa (Sm). Total magnification 200X.

**Figure 5.** Upregulation of type I TNF-α receptor (TNFR1) mRNA expression in gut tissue following delayed bortezomib administration. B6 (H2^b^) recipients of BALB/c (H2^d^) 15 million bone marrow and 20 million spleen cells were treated with or without 15 µg per dose of
bortezomib daily from day +12 through +13. Small intestine and liver were collected at 6 hours after bortezomib administration (day+13) for RNA extraction. TNFR1 mRNA expression was analyzed by RNase Protection Assay (RPA). A and B: Autoradiograph of TNFR1 and GAPDH bands from gut (A) and liver (B) RPA gels. C. Quantitative levels of TNFR1 steady state mRNA levels in the gut and liver tissue from bortezomib and vehicle control treated animals. Quantitative levels of TNFR1 mRNA were determined by densitometric analysis and are expressed as a ratio of the band volumes of TNFR1 normalized to the GAPDH housekeeping gene. These data are representative of 2 (liver) and 4 (gut) independent experiments. N.S: Not statistically significant.

**Figure 6.** Delayed bortezomib administration significant increases serum cytokine levels in mice with GVHD. B6 (H2b) recipients of BALB/c (H2d) 15 million bone marrow cells with or without 20 million spleen cells (SC) were treated with or without 15 µg per dose of bortezomib daily, day 12-13 post-BMT. Serum was collected at 6 hours after bortezomib administration (day+13). Significant increases in TNF-α, IL-1β and IL-6 were observed in delayed bortezomib-treated recipients with SC compared with vehicle control-treated recipients with SC ($P < 0.05$). Representative data from one of three independent experiments are presented, 5 serum samples per group.
Figure 1

A

Bortezomib day0-2
No Bortezomib
Bortezomib day5-7

Proportion Surviving

Days Post-BMT

B

Bortezomib day0-2
No Bortezomib
Bortezomib day0-2 & day5-7

Proportion Surviving

Days Post-BMT

C

Bortezomib day 0
Bortezomib day 5
Bortezomib day 12
No Bortezomib

Proportion Surviving

Days Post SC Infusion
Figure 2

A, B, C, D, E: Graphs showing percent survival over days post-BMT for different conditions involving SC injections and Bortezomib treatments.
Figure 3

Small Intestine

Colon

Liver

P < 0.001

P < 0.0001

N.S.
Figure 4
Figure 5

A

1 2 3 4 5 6 7 8

TNFR1

GAPDH

Lanes 1 - 4: Vehicle control
Lanes 5 - 8: Delayed bortezomib administration

B

1 2 3 4 5 6

TNFR1

GAPDH

Lanes 1-3: Vehicle control
Lanes 4-6: Delayed bortezomib administration

C

Small Intestine TNFRI

Liver TNFRI

Volume Ratio (GAPDH)

P< 0.005

N.S.
Figure 6

**Serum TNF-α (pg/mL)**

- SC, No Bortezomib
- SC, day 12-13 Bortezomib
- No SC, day 12-13 Bortezomib

3/5 samples < 3pg/ml undetectable

**Serum IL-1β (pg/mL)**

- SC, No Bortezomib
- SC, day 12-13 Bortezomib
- No SC, day 12-13 Bortezomib

5/5 samples < 20pg/ml undetectable

**Serum IL-6 (pg/mL)**

- SC, No Bortezomib
- SC, day 12-13 Bortezomib
- No SC, day 12-13 Bortezomib
Differential effects of proteasome inhibition by bortezomib on murine acute graft-versus-host disease (GVHD): delayed administration of bortezomib results in increased GVHD-dependent gastrointestinal toxicity

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