Corrections

NEUROSCIENCE. For the article “Fluorometric measurements of conformational changes in glutamate transporters,” by H. Peter Larsson, Anastassios V. Tzingounis, Hans P. Koch, and Michael P. Kavanaugh, which appeared in issue 11, March 16, 2004, of Proc. Natl. Acad. Sci. USA (101, 3951–3956; first published March 4, 2004; 10.1073/pnas.0306737101), the authors note that, due to a typographical error, several of the rates in Fig. 4A are incorrect. The corrected figure and its legend appear below.

BIOPHYSICS. For the article “Preferred peptide backbone conformations in the unfolded state revealed by the structure analysis of alanine-based (AXA) tripeptides in aqueous solution,” by Fatma Eker, Kai Griebenow, Xiaolin Cao, Laurence A. Nafie, and Reinhard Schweitzer-Stenner, which appeared in issue 27, July 6, 2004, of Proc. Natl. Acad. Sci. USA (101, 10054–10059; first published June 25, 2004; 10.1073/pnas.0402623101), the authors note that “Trp” incorrectly appeared as “Try” in Fig. 2. The corrected figure and its legend appear below.

IMMUNOLOGY. For the article “Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib,” by Kai Sun, Lisbeth A. Welniak, Angela Panoskaltsis-Mortari, Matthew J. O’Shaughnessy, Haiyan Liu, Isabel Barao, William Riordan, Raquel Sitcheran, Christian Wysocki, Jonathan S. Serody, Bruce R. Blazar, Thomas J. Sayers, and William J. Murphy, which appeared in issue 21, May 25, 2004, of Proc. Natl. Acad. Sci. USA (101, 8120–8125; first published May 17, 2004; 10.1073/pnas.0401563101), the authors request that the following error be noted. In the description of “In Vivo Studies” in Materials and Methods, the dose of total body irradiation should have been stated as 900 cGy for experiments performed at the University of Nevada, Reno. The article inadvertently indicated the dose as 1,300 cGy. The dose range of 900–950 cGy for experiments performed at the National Cancer Institute was correctly listed.

Fig. 4. An EAAT3 model with only three conformational changes simulates our experimental findings. (A) A modified 15-state model (16, 28) used to simulate the fluorescence data. The steady-state fluorescence data are well described by the 15-state model, after assigning three fluorescence intensities (2.4, 1.24, and 4) to the different states indicated by the dashed boxes. (B) Simulation of Δf-V in different external solutions: 100 mM Na+ (□), 100 mM choline (□), 100 mM K+ (□), and 100 Na+ mM plus 1 mM glutamate (○). (C) Simulation of Δf-V in different external Na+ concentrations: 50 mM (□), 100 mM (□), 200 mM (□) (pH external 7.5). (D) Simulation of Δf-V at different external pH: pH 6.5 (□), pH 7.5 (○), and pH 8.5 (□); Na+ external = 100 mM. (E) Simulated reverse transport current. The steady-state reverse transport current at 0 mV is plotted at different pH (both internal and external were altered in parallel): K+ internal = 50 mM, K+ external = 50 mM, Na+ internal = 25 mM, Na+ external = 25 mM, glutamate internal = 10 mM, and glutamate external = 0.1 μM.

Fig. 2. Representation of the dihedral angles obtained for the investigated AXA peptides in the upper left square of the Ramachandran space. The Zimmerman code was used to differentiate between the conformational regions of extended β-strand and PPII. The dotted and solid vertical lines are from the transition region discussed in the text.

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Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib


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Graft-versus-host disease (GVHD) represents a major hurdle impeding the efficacy of allogeneic bone marrow transplantation (BMT). Bortezomib is a proteasome inhibitor that was recently approved for treatment of myeloma. We found that bortezomib potently inhibited in vitro mixed lymphocyte responses and promoted the apoptosis of alloreactive T cells. Bortezomib given at the time of allogeneic BMT in mice resulted in significant protection from acute GVHD. Reductions in GVHD-associated parameters and biological evidence of proteasome inhibition were observed with this regimen but with no adverse effects on long-term donor reconstitution. Assessment of graft-versus-tumor responses in advanced leukemia-bearing mice demonstrated that only the combination of allogeneic BMT and T cells with bortezomib promoted significant increases in survival. Increased cytotoxic T cell killing of the tumor was also observed. Thus, the combination of proteasome inhibition with selective immune attack can markedly increase the efficacy of BMT in cancer.

The clinical use of allogeneic bone marrow transplantation (BMT) for cancer treatment is seriously hampered by the occurrence of graft-versus-host disease (GVHD). For the most part, efforts to reduce the incidence of GVHD have also diminished graft-versus-tumor (GVT) responses with increased tumor relapse, suggesting that these two processes are intimately linked (1). GVHD is an immune-mediated disease in which donor T cells recognize and attack the genetically disparate recipient. It has a complex pathophysiology, ultimately involving many different cell types at the final effector stage and it affects multiple organs (2–4). Cytokines, in particular, inflammatory cytokines produced by the T cells and other immune cells, have multiple organs (2–4). Cytokines, in particular, inflammatory cytokines produced by the T cells and other immune cells, have been shown to be critical for GVHD generation and play an important role in fueling the entire process (5–12). The conditioning of the recipient by irradiation or chemotherapy contributes to the production of inflammatory cytokines and has a dramatic impact on GVHD pathophysiology (13). The transcription factor NF-κB has been demonstrated to play a pivotal role in cytokine signaling and the generation of cell-mediated immune responses in numerous models (14–16). In addition, the proteasome has been shown to play critical roles in T cell activation, proliferation, and apoptosis (17), in part, because of blockade of NF-κB activation (15, 18).

The proteasome inhibitor bortezomib (Velcade, formerly PS-341) has been demonstrated to exert numerous biological effects that include blocking the activation of NF-κB (19). Bortezomib, a boronic acid dipeptide derivative, is a member of a class of antitumor compounds and has recently been approved as a single agent for the therapy of multiple myeloma because of its direct growth-inhibitory and apoptotic effects on this cancer (20, 21). Bortezomib has also been shown to have antitumor effects either alone or in combination with chemotherapeutic agents against numerous other tumors (22–24). We have recently demonstrated that bortezomib can sensitize both murine solid tumor and leukemia cell lines to apoptosis by tumor necrosis factor-related apoptosis-inducing ligand (25). Thus, bortezomib is a potentially attractive candidate for administration after BMT because of its direct antitumor effects and its ability to sensitize tumor cells to immune-mediated death pathways, in addition to its potential ability to inhibit alloreactive T cell expansion during GVHD.

The results presented in this study demonstrate that systemic proteasome inhibition can significantly inhibit acute lethal GVHD and preserve GVT responses in advanced tumor-bearing mice after allogeneic BMT with no observed adverse effects on myeloid recovery and donor chimerism.

Materials and Methods

Animals. Female BALB/c (H2b) and C57BL/6 (B6, H2a) mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Mice were between 8 and 13 weeks of age at the start of the experiments.

Cell Lines and Reagents. C1498 (H2b) murine leukemia cell line was maintained as described (25). The proteasome inhibitor bortezomib was kindly provided by Millennium Pharmaceuticals (Cambridge, MA).

In Vivo Studies. B6 mice received myeloablative doses of total body irradiation from a 137Cesium source, based on previously determined irradiation studies at each of the two animal facilities where experiments were performed (900–950 cGy at the National Cancer Institute; 1,300 cGy at the University of Nevada, Reno). Irradiation was followed by the infusion of 1.5 × 107 BALB/c bone marrow cells i.v. with or without BALB/c splenocytes (SCs; 2–4 × 107 cells i.v.) as a source of alloreactive T cells or 1.5 × 107 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled purified splenic T cells. Mice then received Dulbecco’s PBS, or bortezomib in PBS with a dose range of 10–20 mg/kg from days 0 through +3 post-BMT, with comparable results. Mice received sulfamethoxazole/trimethoprim oral suspension USP (Alpharma USPD, Baltimore) in their drinking water beginning 7 days before the transplant.

In the GVT studies, C1498 tumor cells (2 × 105 cells) were...
Injected i.v. into B6 mice 10 days before lethal total body irradiation. BMT was performed as described above. Mice were monitored and weighed weekly. Moribund mice were killed, and organs were subjected to pathological examination to determine the cause of death. All experiments were performed at least three times with 8–11 mice per group.

**In Vitro Mixed Lymphocyte Reaction (MLR) Cultures.** To prepare responder BALB/c T cells, lymph node cells were enriched by passage through a goat anti-mouse and goat anti-rat-Ig-coated column (Cedarlane Laboratories). Stimulator cells were prepared from single-cell suspensions of spleens from B6 mice that were anti-Thy 1.2 mAb (clone 30H-12) and anti-NK1.1 mAb (clone PK136) plus rabbit complement-treated and irradiated (30 Gy). Responders and stimulators were cultured at a final density of 10^6 cells per ml, pulsed with tritiated thymidine (1 μCi per well) (Amershams Pharmacia Life Sciences) 16–18 h before harvesting and counted in the absence of scintillation fluid on a β-plate reader (Packard). Five individual wells were analyzed per data point.

**CFSE Labeling.** Purified T cells were labeled with 1–2.5 μM CFSE (Molecular Probes) according to the manufacturer’s instructions.

**Flow Cytometry.** Leukocytes were labeled as described (26). In brief, 10^6 cells were labeled with antibody for two- or three-color flow cytometry by using CFSE, FITC, phycoerythrin, PerCP, or biotin- (along with SA-PerCP) conjugated mAbs purchased from BD Biosciences (San Diego). Nonspecific binding was corrected with isotype-matched controls. All results were obtained by using a either a FACSCalibur or a FACSscan (Becton Dickinson).

**Cytokine Analysis.** Serum levels of cytokines were determined by multiplex analysis based on a Luminex (Austin, TX) by using mouse cytokine-specific bead sets and standards according to the manufacturer’s instructions (R & D Systems).

**Cytotoxicity Assay.** For the generation of cytotoxic T cells (CTLs), BALB/c mice were immunized with 10^7 irradiated (100 Gy) C1498 cells by i.p. injection, twice a week for 2 weeks, and spleens were harvested 2 weeks later. CTLs were restimulated with irradiated C1498 for 5 days in vitro. Two thousand viable C1498 cells were then incubated with CTL, at different effector-to-target ratios with or without 4 nM bortezomib for 36–45 h. The level of cytotoxicity was determined by annexin V binding and propidium iodide incorporation and clonogenic tumor cell growth in semisolid media as described (27).

**Measurement of 20S Proteasome Inhibition.** Tissue homogenates were prepared from whole spleens of treated animals. Kinetic measurement of proteasome activity has been described (28, 29). Details and calculation for specific activity are available in Supporting Text, which is published as supporting information on the PNAS web site.

**Electromobility Shift Assays.** Preparation of nuclear and cytoplasmic extracts and subsequent electromobility shift assays were performed as described (30). Details are described in Supporting Text.

**Statistics.** Survival data were plotted by the Kaplan–Meier method and analyzed by the log-rank test. In vitro assays were analyzed by ANOVA and the unpaired Student t test as indicated. A P value of <0.05 was considered significant.

**Results**

**Bortezomib Can Inhibit MLRs in Vitro.** Bortezomib has been demonstrated to potently inhibit proteasome activity both in vitro and in vivo. It also blocks degradation of IκB, leading to impaired NF-κB activation (21), an important factor in both cytokine responsiveness and maturation of functional cell-mediated immune responses. Therefore, we assessed the effects of bortezomib on alloreactive MLR responses in vitro. The results demonstrate that bortezomib is capable of significantly inhibiting proliferation of alloreactive T cells in a dose-dependent manner (Fig. 1A). In an MLR assay, assessment of cell division by CFSE dye dilution and annexin V binding as a marker of apoptosis demonstrated that significant increases in apoptosis of alloreactive CD4⁺ and CD8⁺ T cells occurred on day 6 in the presence of 4 nM bortezomib (Fig. 1 B and C). Apoptotic cells were predominately in the CFSElo population, suggesting that the proliferating T cells were primarily targeted. These results indicate that bortezomib is capable of potently suppressing T cell responses, in part, through promotion of apoptosis of activated T cells. Reductions in proliferative capability, as indicated by decreases in the number of CFSEhi cells with increasing amounts...
of bortezomib, were also observed (data not shown). Thus, bortezomib was capable of both inhibiting cell proliferation and selectively inducing apoptosis in the activated alloreactive T cell population in vitro.

**Bortezomib Administration Protects Mice from Acute Lethal GVHD.** Because bortezomib significantly suppressed alloreactivity in vitro, we next assessed its effects in vivo by using a murine model of acute lethal GVHD. C57BL/6 mice received a myeloablative dose of irradiation followed with allogeneic bone marrow cells and SCs from full MHC major and minor antigen disparate BALB/c donors. With this regimen, all mice succumbed to severe acute GVHD affecting the gut and liver within 10 days (Fig. 2A). However, when bortezomib (10 μg per dose) was administered daily on days 0 through +3 post-BMT, significant (P < 0.0001) increases in survival were observed. In this model, all mice eventually succumbed to acute GVHD although at a much later time point after bortezomib treatment (median, day +70 post-BMT). The level of protection from GVHD by bortezomib was even greater (100% survivors, day +100 post-BMT) when the number of donor splenocytes was decreased to reduce the severity of the GVHD in which all control mice succumbed within 40 days (Fig. 2B). In these experiments, the administration of bortezomib resulted in 100% long-term survival. These results demonstrate that early administration of bortezomib is capable of preventing the occurrence of acute lethal GVHD after allogeneic BMT in mice. Similar protection from GVHD was observed when the dose of bortezomib was changed to 20 μg given at day 0 and +2 post-BMT (data not shown). The administration of bortezomib did not significantly affect either platelet or white blood cell recovery or long-term donor chimism after allogeneic BMT (Fig. 7, which is published as supporting information on the PNAS web site). When the mice were assessed for the effects on donor T cell engraftment and proliferation 3 and 4 days after BMT, it was found that administration of bortezomib resulted in significantly less donor CD4+ and CD8+ cell engraftment (Fig. 3A) due to decreases in proliferating T cells (data not shown) with a significantly (P < 0.001) higher proportion of annexin V binding on this population, indicating increased apoptosis of alloreactive T cells (Fig. 3B) similar to the effects observed in vitro (Fig. 1B and C). Donor T cell chimism was also not adversely affected by bortezomib treatment. Mice that received BMT and donor splenocytes and either bortezomib or control had >90% donor T cells at day 15 and >99% donor T cells at day 30 after induction of moderate GVHD. Serum tumor necrosis factor α (TNF-α) levels assessed after BMT indicated that administration of bortezomib resulted in significantly (P < 0.05) reduced TNF-α levels at both day 7, during the period of T cell activation and expansion, and day 30 (Fig. 3C) after BMT, correlating with reduced GVHD in treated animals. Thus, the protection from acute lethal GVHD is associated with an initial reduction of donor T cell chimerism, increased alloreactive T cell apoptosis, and reductions in systemic TNF-α after bortezomib treatment.

**Mechanism of Bortezomib Action in Vivo.** Bortezomib has been shown previously to affect the activation and nuclear translocation of NF-κB by blocking the function of the 20S proteasome. We next determined whether these mechanisms were involved in the in vivo activity of bortezomib after BMT. The activity of NF-κB in nuclear lysates was assessed by electromobility shift assays on splenocytes recovered day +4 post-BMT. By day +4 a 45% reduction had occurred in activity of NF-κB in the nucleus of splenocytes from mice treated with bortezomib compared with PBS (Fig. 4A; P < 0.05). Mice were killed on days +3 and +4 post-BMT when the enzymatic activity of the 20S proteasome in spleen cells was quantified. A 31% reduction in 20S proteasome activity was observed in the splenocytes of mice after treatment with bortezomib on day +3, which decreased to 15% by day +4 post-BMT (Fig. 4B). This finding suggests that systemic administration of bortezomib at this dose and regimen results in reduction of function of the 20S proteasome and also leads to diminished nuclear activity of NF-κB in vivo.

**Bortezomib Preserves GVT Responses after Allogeneic BMT in Advanced Tumor-Bearing Mice.** Although preventing GVHD is an important step in improving the efficacy of BMT, it is imperative to also evaluate effects on GVT, because many agents that curtail GVHD also suppress GVT responses. Because bortezomib can directly mediate antitumor effects in vitro and in vivo, it seemed likely that antitumor responses might be maintained after allogeneic BMT and bortezomib treatment. The C1498 murine acute myeloid leukemia line was found to need higher amounts (10–20 nM) of bortezomib to directly inhibit growth in vitro (25) compared with ranges affecting MLR responses (3–4 nM) (Fig. 1A). We first treated advanced-tumor-bearing mice with bortezomib alone at a dose range that was found to inhibit GVHD (15 μg per dose). When the mice were treated 10 days after receiving the tumor, no protective effects of bortezomib alone were observed in these advanced-tumor-bearing recipients (Fig. 5A). Modest increases in survival were observed when mice received allogeneic BMT alone (P < 0.005; Fig. 5B) and the addition of bortezomib administration did not result in significant increases in survival compared with mice that received an
allogeneic BMT alone by using this dose and regimen (Fig. 5B). When the tumor-bearing mice received allogeneic BMT with donor splenocytes, all mice rapidly succumbed to GVHD (Fig. 5C). Only the tumor-bearing mice that received an allogeneic BMT with donor splenocytes and bortezomib demonstrated significant increases in survival (P < 0.0001; Fig. 5C). In agreement with the GVHD data, mice receiving these higher numbers of spleen cells and bortezomib eventually succumbed to GVHD rather than tumor, indicating that significant GVT effects had been obtained. When mice were given lower numbers of spleen cells with bortezomib, no significant antitumor effects were observed as the mice all succumbed to tumor because of the extensive tumor burden at the start of the treatment (data not shown). These results demonstrate that bortezomib can protect from GVHD and preserve GVT effects after allogeneic BMT in advanced tumor-bearing mice.

It has been shown that bortezomib can synergize with tumor necrosis factor-related apoptosis-inducing ligand in the killing of tumor cells (25); therefore, we explored the possibility that the T cells capable of mediating GVT in vivo could also mediate increased killing of the tumor after bortezomib administration. In these studies, C1498 leukemia cells were coincubated with allogeneic CTL in the presence or absence of bortezomib in vitro. The data show that the addition of bortezomib, at dose levels that do not directly affect tumor cell viability, does indeed result in increased CTL killing of tumor cells in vitro (Fig. 6). Significant increases in tumor cell apoptosis, decreases in total viable leukemia cells recovered (Fig. 6 A and B), and decreases in clongeneic leukemia cell growth (Fig. 6C) were observed when bortezomib was cocultured with CTL and the tumor. This result represents another potential pathway by which bortezomib can promote GVT effects in vivo, i.e., by sensitizing tumor cells to immune-mediated destruction. Therefore, the administration of bortezomib early after BMT can maintain or augment the GVT response and protect from GVHD, indicating that these two
biological responses are influenced to differing degrees after this treatment.

Discussion

Dissociation of GVHD and GVT remains of paramount importance in improving the efficacy of BMT. The data presented here demonstrate that proteasome inhibition by using bortezomib can markedly prevent acute lethal GVHD yet still retain GVT responses in advanced tumor-bearing mice. These results suggest that bortezomib may be of significant value for removal of the minimal residual disease that can exist after BMT. That bortezomib has also been shown by us and others to affect solid tumors as well would suggest that the combined use of proteasome inhibition with bortezomib and allogeneic BMT may be of use in the therapy of both blood-borne and solid cancers.

Proteasome inhibition by bortezomib has the potential to provide numerous antitumor effects, both direct and indirect. Although many effects of bortezomib on numerous neoplastic cells have been presumed to be due to blockade of NF-kB, the leukemia cell line used in our previous studies has been shown to resist the inhibitory effects of bortezomib on NF-kB (25). This finding suggests that even greater antitumor effects could be anticipated on tumor cells that are additionally sensitive to the blockade of this pathway. Promotion of GVT responses were observed only when both bone marrow and SCs were administered. Therefore, in this model, it is likely that the major role of bortezomib is to potentiate immune-mediated tumor clearance rather than directly inhibit tumor cell growth.

We recently reported that bortezomib can sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated killing, in part, because of cellular reductions in cellular FLICE inhibitory protein (25). This finding would also suggest that other cytolytic pathways (i.e., Fas ligand/Fas and TNF/TNFR1) may be enhanced after exposure of tumor
cells to bortezomib. Because bortezomib can sensitize the tumor cells to T cell-mediated killing, the cell-mediated GVT effects that follow BMT, or with delayed lymphocyte infusion, may not only be spared but actually enhanced after bortezomib administration.

The ability of bortezomib to prevent GVHD was predicated on its administration immediately after BMT. Although no adverse effects or toxicities were observed by using this regimen, later administration of bortezomib while acute GVHD was ongoing can also increase GVHD if administered later when GVHD is actively ongoing (31–33).

The mechanism by which proteasome inhibition by bortezomib prevents GVHD is not definitively known, although a likely candidate would be inhibition of the NF-κB pathway. This transcription factor has been demonstrated to be critical for cytokine responsiveness and development of cell-mediated responses (15, 34). The suppression of GVHD may be due, in part, to a reduction of cytokine production concurrent with the suppression of apoptosis of the alloreactive T cells. The reductions in serum TNF-α levels would also suggest that a blunting of the cytokine production may be occurring. The demonstration that increased apoptosis of alloreactive T cells can occur after exposure to bortezomib suggests that this may also be an important contributing mechanism. It is possible that bortezomib rapidly induces the preferential deletion of the very-high-affinity alloreactive T cells (as suggested by the in vitro MLR and in vivo CD4+ and CD8+ cell engraftment data), thus allowing for the expansion of the remaining T cells that maintain GVH responses yet have a reduced potential for promoting GVHD. Although it will be of interest to compare the efficacy of agents specific for blockade of NF-κB with bortezomib, it may be difficult to definitively delineate the mechanism(s) of action of bortezomib on GVHD protection and GVT promotion because of the differing efficiencies of these agents in blocking NF-κB in vivo.

In summary, the data presented here demonstrate that proteasome inhibition with bortezomib may be of significant use in preventing GVHD and preserving or possibly even promoting GVT responses in cancer. The models in which advanced tumor-bearing mice were used suggests that this therapeutic approach may be of use to remove the minimal residual disease that can exist after extensive conditioning by selectively predisposing the tumor cells to immune-mediated attack. Further, proteasome inhibition may also be of significant use for the suppression of T cell responses after solid organ transplantation or during autoimmune diseases.

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