A Small Interference RNA Screen Revealed Proteasome Inhibition as Strategy for Glioblastoma Therapy

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Glioblastoma (GBM) is the most common cancer of the central nervous system (CNS).[30,41] Despite advances in our understanding of its molecular pathogenesis, GBM remains a devastating disease. The current standard of care for GBM consists of surgical resection followed by combined radiation therapy and temozolomide (TMZ) chemotherapy.[52] Unfortunately, this regimen provides only palliative relief because nearly all patients die of the disease within 3 years of diagnosis.[33,41,52]

One strategy for developing new GBM therapeutics is to build on the modest efficacy of ionizing radiation (IR) and TMZ. Traditionally, this is achieved by screening a large number of chemicals to identify subsets that enhance the tumor-killing effect of IR and TMZ.[23] Typically, these subsets of chemicals exhibit limited biological activity and require structural refinement before acceptable efficacy can be achieved. Because of the potentially infinite number of derivatives that can be made from a single candidate chemical, this refinement process is laborious and time-consuming. To expedite this refinement process, molecular targets of the candidate chemical are frequently sought. Unfortunately, because pharmacological agents inevitably affect multiple molecular targets, identification of these targets often requires years of investigation.

Recent development of small interference RNA (siRNA) technology now allows identification of molecular targets critical for mediating specific biological processes.[18] This capability revolutionizes drug development by allowing for molecular target discovery followed by the identification and synthesis of compounds that selectively inactivate the target. siRNA is a class of short double-stranded RNA molecules (20–30 nucleotides in length) that interferes with gene expression in a sequence-specific manner. On transfection into mammalian cells, siRNAs are processed by an enzyme called DICER and assembled into a complex known as RISC (RNA-inducing silencing complex). This complex uses the siRNA sequences as a means to identify and degrade complementary mRNA sequences,[35] thereby preventing protein synthesis and subsequent phenotypic expression. With the complete sequencing of the human genome,[11] it is now possible to design siRNA specific to each gene to determine its effect on any biological process of interest.

Because both IR and TMZ exert their tumoricidal effect by the induction of DNA damage beyond cellular capacity for repair,[17,49] we hypothesized that silencing selected DNA repair genes may augment tumor killing by these agents. We therefore screened a commercially available siRNA library targeting the 356 genes previously implicated in DNA repair. We found that genetic silencing of PSMA1 (prosome, macropain subunit, alpha type, 1), a critical component of the proteasome,[16,31,40,61] strongly sensitized the U87MG cell line to both TMZ and IR.

The proteasome consists of a multisubunit protein complex that degrades proteins by coordinated activities of chymotryptic, tryptic, and postglutamyl peptidases.[40,42] The proteasome complex is the major mechanism of intracellular protein degradation.[10] By processing critical proteins involved in DNA repair, cell proliferation, and apoptosis,[40] the proteasome complex indirectly regulates these processes.

Proteasome inhibitors have been shown to cause cell growth arrest and apoptosis in several glioblastoma cell lines.[39,53,60] When treated with lethal concentrations of proteasome inhibitor, glioblastoma cells first develop G2/M arrest before undergoing apoptosis. The G2/M arrest is largely attributable to an accumulation of cyclin B1, p27, and p21, the degradation of which is required for cell-cycle progression.[2,38,58] The signaling mechanism by which G2/M arrest triggered apoptosis remains unclear.

Here we showed that independent proteasome inhibitors sensitized GBM cell lines to IR and TMZ at sublethal concentrations. This sensitizing effect was independent of the cellular p53 status. Importantly, the sensitization was observed in neurosphere cell lines with stemlike properties, suggesting that the combined activity is effective against cancer stem cells. The order of addition is critical because sensitization is achieved only when proteasome inhibition is delayed after TMZ or IR treatment. The mechanism of sensitization is, in part, mediated by modulation of DNA damage response.

METHODS

Cells and Cell Culture Methods

The U87MG, U343MG, and U373MG cell lines were obtained from American Type Culture Collection (Manassas,

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VA). The U87MG EGFRvIII,[36] U87MG DN p53,[1] BT69,[62] BT74,[62] BT75,[62] and BT78[62] lines were generated as previously described. U87MG H2B-GFP was kindly provided by Dr. Yoshinaga Saeki (Ohio State University). Non-neurosphere cell lines were propagated at 37°C (humidified atmosphere containing 5% CO_2) in Dulbecco's modified Eagle medium (Gibco, Rockville, MD) supplemented with 15% fetal calf serum (Sigma Aldrich, St. Louis, MO), 2 mM L-glutamine, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Gibco). Neurosphere cell lines were serially propagated by subcutaneous implantation in NOD-SCID mice and harvested only for TMZ and IR sensitivity assays. For these assays, the tumors were harvested when they reached approximately 1 cm in size. Tumors cells were disaggregated, counted, and then grown in a serum-free media with epidermal growth factor, fibroblast growth factor, and leukemia inhibitory factor as previously described.[29,50]

TMZ (AK Scientific, Mountain View, CA) was dissolved in deionized water immediately before use. Velcade (bortezomib) (AK Scientific, Inc., Mountain View, CA) and lactacystin (EMD Chemicals, Gibbstown, NJ) were dissolved in deionized water. ALLN (EMD Chemicals) was dissolved in DMSO (Sigma Aldrich). To test the sensitizing effects of the proteasome inhibitors, each inhibitor was titrated in terms of cytotoxic effects. The concentration of Velcade (5 nM), ALLN (1 μM), and lactacystin (10 nM) were selected to ensure 80% to 90% cell survival. The concentration of TMZ (100 μM) was selected to achieve 30% to 50% cell kill.

For the adherent cells (U87MG, U87MG DN p53, U373MG, U343MG, and A172), experiments involving Velcade, ALLN, or lactacystin were done in the following manner. Cells were treated with the indicated concentration for 24 hours. The cells were then washed twice with 1x phosphate-buffered saline (PBS) and then incubated in fresh medium. For TMZ, cells were treated for three hours. The cells were then washed twice with 1x PBS and then incubated in fresh medium.

For Velcade pretreatment experiments, cells were treated with 5 nM Velcade for 24 hours. The Velcade-containing medium was then removed, and the cells were washed and treated with medium containing 100 μM TMZ for 3 hours. The TMZ-containing medium was then replaced with fresh medium. For concurrent TMZ/Velcade treatment, cells were treated with medium containing 100 μM TMZ and 5 nM Velcade for three hours. This medium was then removed. The cells were washed and incubated for an additional 21 hours with medium containing 5 nM Velcade. This medium was then replaced with fresh medium. For the delayed Velcade treatment, cells were incubated in 100 μM TMZ-containing medium for three hours. This medium was replaced with fresh medium, and the cells were incubated for an additional 21 hours. After this incubation period, Velcade was added to a final concentration of 5 nM. The cells were incubated in this medium for another 24 hours. The Velcade-containing medium was then replaced with fresh medium.

Because the neurospheres were nonadherent, serial washes and medium changes were not practical. As such, the protocol for the neurosphere experiments was modified to the following. The neurospheres were treated with various concentrations of TMZ for 24 hours. Velcade was then added to a final concentration of 1 nM. The cells were incubated in this mixture for seven days before viability determination by the MTT Viability Assay kit (Biotium, Hayward, CA).

siRNA Screening

Transfection of cell lines in a 96-well format with the QIAGEN DNA repair library was performed as previously described.[26] For the radiation sensitization screen, the cells were irradiated with 5 Gy of IR 24 hours after transfection using the Gamma40 (cesium-137 source) irradiator (Best Theratronics Ltd, Ottawa, Canada). For the TMZ sensitization screen, the cells were treated with 50 μM of TMZ 24 hours after transfection. The IR and TMZ doses were empirically determined to achieve 50% to 70% cell kill in our assay. Viability was assessed five days after treatment using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI). At this time point, the cells were approximately 60% to 80% confluent in each well. The experiment was performed twice for each cell line to allow for statistical analysis. Statistical analysis was performed as previously described.[26] In brief, the corrected viability for each siRNA oligonucleotide was calculated as a percentage of the mean viability of the 16 control wells on each plate. The corrected viability of the treated cells was then divided by the corrected viability of the nontreated cells to calculate the relative viability after irradiation for each respective gene target. The mean viability of the treated cells relative to the nontreated cells (also referred to as the mean percentage of viability after treatment), along with the standard deviation, was calculated from four individually corrected viability values (averaging the two distinct siRNA oligonucleotides directed against the same target gene in each of the two experiments). The candidates were ranked based on the mean percentage of viability after treatment. The sequences of the siRNAs are available on request.

Viability Assays

For the neurosphere assay, the MTT Viability Assay kit (Biotium) was used in accordance to the manufacturer's instructions. For clonogenic assays, the cells were seeded in 10-cm dishes and treated with the various concentrations of Velcade, TMZ, and IR in the orders as described previously. Colonies were counted after crystal violet staining at 14 days.[7]

Flow Cytometry

U87MG cells (1×10^5) were plated onto 6-well plates and cultured overnight. The drug treatment was done as

indicated in *Figure 19.2B*. The cells were harvested at each time point and fixed with 70% ethanol and stored at −20°C. After the collection of all the samples, the cells were washed with PBS twice, treated with 1 mg/mL of RNAseA/PBS for 15 minutes, and stained with propidium iodide (25 μg/mL). Fluorescence-activated cell sorting was performed with FACSCalibur (BD Biosciences), and cell cycle distribution was analyzed with CellQuest software (BD Bioscience, San Jose, CA); 1×10^4 cells were analyzed for each sample.

Live Cell Imaging

U87MG stably expressed H2B-GFP cells were grown on 12-well glass-bottom dishes (MatTek, Ashland, MA) overnight (5×10^4 cells per well). Drug treatment was done as described in *Figure 19.2B*. Images were acquired automatically from each well using a Nikon TE2000E PFS inverted microscope fitted with a 20× Nikon Plan Fluor objective (Nikon, Melville, NY), a linearly encoded stage (Prior ProScan, Prior Scientific, Rockland, MA) and a Hamamatsu Orca-ER CCD camera (Hamamatsu, Bridgewater, NJ). The microscope was controlled using NIS Element (Nikon). The microscope was housed in a custom-designed 37°C chamber with a secondary internal chamber that delivered humidified 5% CO_2. Fluorescence and differential interference contrast images were obtained every 15 minutes for a period of 72 hours.

For analysis of abnormal mitosis, cells were analyzed if they entered mitosis at least 20 hours before the end of imaging. Mitotic chromosomes were analyzed for the presence of chromosome segregation error. We also documented whether cells completed cytokinesis. The cells were scored as having chromosome segregation error if H2B-GFP–positive chromatin was found outside its mother nucleus during anaphase/telophase. Cells were considered to be in prolonged mitotic arrest if they arrested for more than 24 hours.

Mouse Xenograft Model and In Vivo Imaging

The U87 cell line harboring a luciferase expression construct was constructed as previously described.[43] Cells derived from this cell line were harvested in mid-logarithmic growth phase and resuspended in PBS. Homozygous NCR nude mice (Charles River, Wilmington, MA) were anesthetized with ketamine hydrochloride at 150 mg/kg and xylazine at 12 mg/kg (Phoenix Pharmaceuticals, St. Joseph, MO) intraperitoneally before head fixation in a stereotactic frame (Stoelting, Wood Dale, IL). The cranium was exposed. One hundred thousand U87-Luciferase cells were resuspended in 10 μL of PBS and injected through a 27-gauge needle for two minutes at 2 mm lateral and posterior to the bregma. The depth of the implant was 3 mm below the dura. The incision was closed with Vetbond (3M Co., St Paul, MN).

Uptake of the implanted U87 cells were assessed 15 days after implant using in vivo imaging (see below). The mice with successful uptake were divided into four groups, with four to five mice in each group. The TMZ-only group was treated with 15 mg/kg TMZ (orally) on days one, two, and three. The Velcade-only group was treated with 1 mg/kg of Velcade (intravenously) on day one. The TMZ + Velcade group was treated with 15 mg/kg of TMZ (orally) on days one, two, three and 1 mg/kg of Velcade (intravenously) on day five. Weekly in vivo imaging was performed thereafter. The control group was mock-treated with vehicle medium at the time points described above.

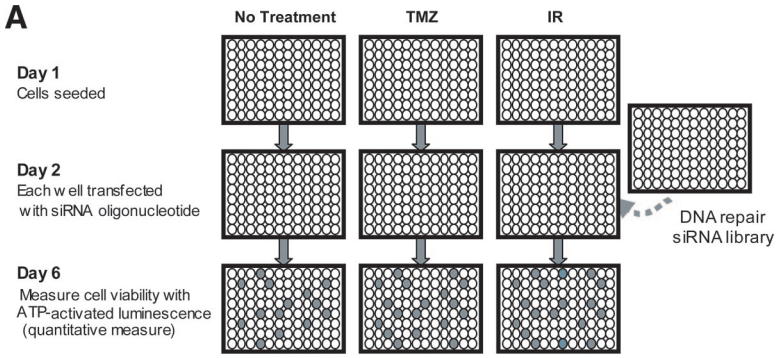
For in vivo imaging, the mice were anesthetized as described above and injected with D-luceferin at 50 mg/mL intraperitoneally (Xenogen, Alameda, CA) and imaged with the IVIS Imaging System (Xenogen) for 10 to 120 seconds, bin size 2. To quantify bioluminescence, identical circular regions of interest were drawn to encircle the entire head of each animal, and the integrated flux of photons (photons per second) within each region of interest was determined using the LIVING IMAGES software package (Xenogen). Data were normalized to bioluminescence at the initiation of treatment for each animal. The experiment was repeated twice.

RESULTS

PSMA1 Silencing Sensitized U87MG to Both TMZ and IR

We selected the U87MG cell line for our screen because of the ease of transfection and gene silencing in this cell line. Because TMZ and IR causes tumor killing by the induction of DNA damage beyond cellular capacity for repair, we reasoned that inactivation of selected DNA repair genes may augment tumor killing by these agents. We used the QIAGEN DNA repair siRNA library that targets 356 DNA repair and damage response genes (for detailed description of the library, please visit http://www1.qiagen.com/default.aspx). All critical genes involved in the major DNA repair pathways and damage response pathways known to date are included in this collection.[26] The library is gridded into a 96-well format (*Fig. 19.1A*) such that each well contains a single siRNA. Each gene target is represented by two distinct siRNAs. The screening process is outlined in *Figure 19.1A* and detailed in the Methods section.

The 15 genes that, when silenced by independent siRNAs, consistently and efficiently sensitized U87MG to TMZ and IR. Many of the gene targets represented by these siRNAs have previously been shown to mediate radiation response (ATM, RAD51L3, ADPRT1, RPA[6,46]) and TMZ response (BRCA1, FANCG, ADPRT2).[7,8,12] The finding that these genes appeared as top sensitizers served to validate our screen. We identified one common gene target that when silenced consistently sensitized U87MG to both TMZ and IR. This gene, PSMA1 (prosome, macropain subunit, alpha type 1), encodes a critical component of the proteasome com-



B

	IR	% viability	SD	TMZ	% viability	st.dev.
1	ADPRT1	0.50	0.10	HRK	0.68	0.07
2	CASP6	0.51	0.11	PRKCG	0.69	0.09
3	CSPG6	0.52	0.20	PTK2	0.69	0.06
4	XAB2	0.53	0.11	H2AFX	0.70	0.14
5	RPA3	0.55	0.10	ARMET	0.70	0.13
6	PSMA1	0.56	0.07	XAB2	0.70	0.07
7	DDX48	0.56	0.09	PIN1	0.70	0.13
8	LMNA	0.57	0.08	PLEC1	0.70	0.11
9	DEPC-1	0.59	0.19	BRCA1	0.70	0.15
10	BUB1B	0.59	0.16	PLK3	0.70	0.08
11	TPX2	0.59	0.08	FBXO5	0.70	0.04
12	RASGRF1	0.60	0.09	ADPRT2	0.70	0.06
13	ATM	0.60	0.05	FANCG	0.71	0.12
14	RAD51L3	0.61	0.08	PLK1	0.72	0.20
15	MSH5	0.62	0.14	PSMA1	0.72	0.09

FIGURE 19.1. Small interference RNA (siRNA) screen to identify gene silencings that sensitized the U87MG line to ionizing radiation (IR) and temozolomide (TMZ). **A,** Schematic depiction of the screen. Cells were seeded into 96-well plates on day one. On day two, each well was transfected with a siRNA oligonucleotide directed against one DNA damage response/repair gene. On day three, one set of cells remained untreated, and another set was treated with TMZ at a concentration of 50 μM. A third set was irradiated with 5 Gy of IR. On day six, the viability of the cells in each well was measured using the Cell Titer-Glo Luminescent Cell Viability Assay kit. ATP, adenosine triphosphate. **B,** The top 15 gene targets from the genetic screen are shown. The percentage of viability and standard deviations were calculated as described in Methods. PSMA1 is indicated in red because silencing of this gene by independent siRNAs sensitized the U87MG line to both TMZ and IR.

plex.[16,31,40,61] This result suggests that proteasome inhibition may be a strategy for simultaneous radiation and TMZ sensitization.

Proteasome Inhibition Sensitized U87MG to TMZ and IR

As a first step to translate the results of our siRNA screen into a clinical strategy, we tested whether proteasome inhibitors sensitized the U87MG cell line to TMZ. As shown in *Figure 19.2A*, Velcade exhibits significant tumoricidal activity in the absence of TMZ or IR treatment at a 10-nM concentration. We therefore selected 5 nM as a sublethal dose for testing TMZ sensitization.

TMZ is an alkylating agent that methylates DNA at a variety of positions.[13,14,17] When unrepaired, these alkylated products cause the formation of DNA strand breaks that ultimately result in cell death or cell senescence.[21,22,51] The process by which alkylated DNA products are converted into DNA strand breaks requires progression through one entire cell cycle.[51] Because Velcade has been shown to induce G2/M arrest,[54] we reasoned that the order of addition between Velcade and TMZ may be critical and tested the various orders of addition.

As shown in *Figure 19.2C*, treatment of U87MG with 5 nM Velcade for 24 hours resulted in 5% to 10% loss in clonogenic survival. Treatment with TMZ for 3 hours caused an approximately 50% reduction in clonogenic survival. Clonogenic survival was not significantly altered by pre- or simultaneous treatment with Velcade. However, delayed Velcade treatment 24 hours after TMZ treatment significantly augmented the tumoricidal activity of TMZ, with clonogenic survival of 5% to 10%. When fit into the Chou-Talalay mutually nonexclusive model,[9] the combination index of this regimen was 0.3, suggesting that the interaction between delayed Velcade treatment and TMZ was synergistic.

Similar results were observed with IR. Pretreatment or simultaneous treatment with Velcade did not significantly augment the tumoricidal activity of IR. Delayed Velcade treatment, conversely, augmented IR-induced tumor killing in a synergistic manner.

We wished to further confirm that the effect of Velcade was entirely due to proteasome inhibition rather than nonspecific activity related to the compound. We therefore tested two other proteasome inhibitors of chemical structure that differ from Velcade (ALLN[47] and lactacystin[24]). As shown in *Figure 19.2D*, the TMZ sensitizing effect of Velcade was recapitulated by ALLN and lactacystin.

TMZ Sensitization by Proteasome Inhibition Is Independent of the Cellular p53 Status

The U87MG line harbors a wild-type p53 gene with an intact p53 axis.[25] Because p53 was previously shown to be an

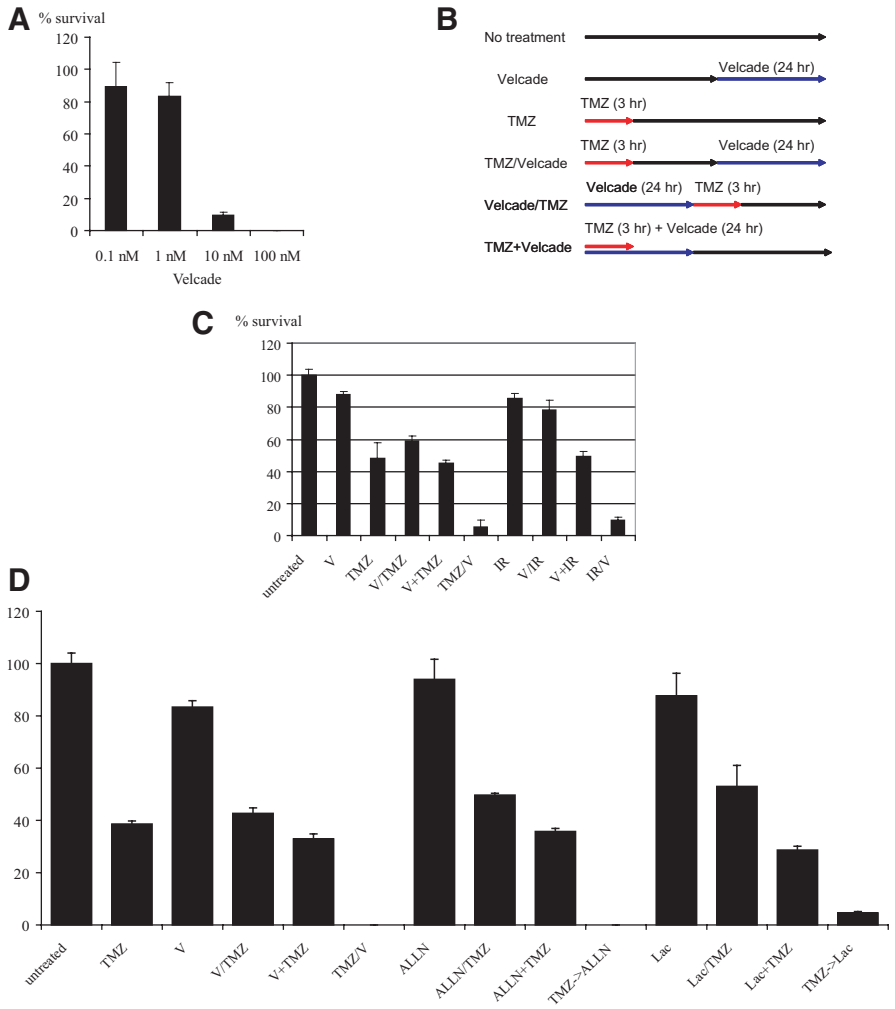


FIGURE 19.2. Delayed Velcade (bortezomib) treatment sensitized the tumoricidal effect of temozolomide (TMZ) and ionizing radiation (IR). **A,** Dose-response curves demonstrating the response of U87MG cells to Velcade. Cells were incubated with the various concentrations of Velcade for 24 hours. The Velcade-containing medium was then removed. The cells were washed twice with 1x phosphate-buffered saline before fresh medium was added. The cells were then incubated in this medium for 14 days before colonies were counted. The percentage of survival after treatment was calculated by dividing viability of treated cells relative to nontreated cells. The results represent the average of triplicates. The experiment was repeated three times. A representative experiment is shown. **B,** Regimen for combining Velcade and TMZ or IR. Control experiments were performed by treating U87MG cells with vehicle (dimethyl sulfoxide), 100 μM TMZ for 3 hours or 5 nM Velcade for 24 hours. For Velcade pretreatment experiments, cells were treated with 5 nM Velcade for 24 hours. The Velcade-containing medium was then removed, and the cells washed and treated with medium containing 100 μM of TMZ for three hours. The TMZ-containing medium was then replaced with fresh medium. For concurrent TMZ/Velcade treatment, cells were treated with media containing 100 μM TMZ and 5 nM Velcade for 3 hours. This media was then removed. The cells were washed and incubated for an additional 21 hours with medium containing 5 nM Velcade. This medium was then replaced with fresh medium. For the delayed Velcade treatment, cells were incubated in 100 μM TMZ-containing medium for 3 hours. This medium was replaced with fresh medium. The cells were incubated for an additional 21 hours in this media. After this incubation period, Velcade was added to a final concentration of 5 nM. The cells were incubated in this media for another 24 hours. The Velcade-containing medium was then replaced with fresh medium. For the IR experiments, the pretreatment, concurrent treatment, and delayed treatment with Velcade were performed in an analogous manner except that TMZ was replaced with IR. **C,** Delayed Velcade treatment sensitized U87MG cells to TMZ and IR. Clonogenic survival assay was performed after treatment described in B. The percentage of survival after treatment was calculated by dividing the viability of treated cells relative to nontreated cells. The results represent the average of triplicates. The experiment was repeated three times. A representative experiment is shown. V, Velcade; V/TMZ, Velcade pretreatment before TMZ; V+TMZ, simultaneous Velcade treatment with TMZ; TMZ/V, delayed Velcade treatment after TMZ. **D,** Independent proteasome inhibitors sensitized U87MG to the tumoricidal effect of TMZ. To confirm that the TMZ-sensitizing effect of Velcade was directly related to proteasome inhibition, we tested the effect of two other proteasome inhibitors (ALLN[47] and lactacystin (Lac)[24]). The treatment regimens were as described for Velcade (**B**) except that ALLN (1 μM) and lactacystin (10 nM) replaced Velcade in these regimens. The percentage of survival after treatment was calculated by dividing the viability of treated cells relative to nontreated cells. The results represent the average of triplicates. The experiment was repeated three times. A representative experiment is shown.

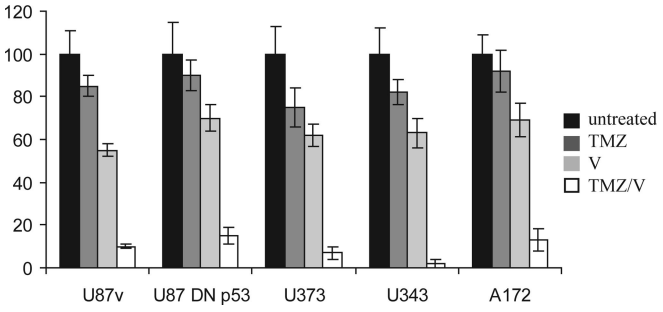


FIGURE 19.3. Proteasome inhibition enhanced tumoricidal effect of temozolomide (TMZ) in both p53 wild-type and p53 mutant cell lines. U343MG and U373MG glioblastoma multiforme (GBM lines) harboring mutated p53. A172 and U87MG are 2 GBM cell lines harboring a wild-type p53. The U87v and U87MG DNp53 cell lines are isogenic except for expression of the DNp53 protein. Treatment regimens are as depicted in *Figure 19.2B*. The percentage of survival after treatment was calculated by dividing viability of treated cells relative to non-treated cells. The results represent the average of triplicates. The experiment was repeated 3 times. A representative experiment is shown. V, Velcade.

important determinant of TMZ and IR response in GBM,[5,15,22] we wished to determine whether the TMZ/Velcade interaction was affected by p53 status. We adapted two strategies to examine this question. First, we compared the effect of the delayed Velcade treatment after TMZ in cell lines harboring wild-type p53 (U87MG and A172) to the effect in GBM lines harboring mutant p53 (U343MG and U343MG). As shown in *Figure 19.3*, all cell lines tested exhibited similar response irrespective of p53 status. We recognize that these four cell lines differ in genetic and epigenetic architecture beyond p53 and that these differences may confound the interpretation of our experiment. We, therefore, tested a U87MG derivative line transfected with a dominant negative p53 allele (U87MG DN p53) and compared this line with a U87MG derivative transfected with an empty vector (U87v).[1] As shown in *Figure 19.3*, the U87MG DN p53 was more resistant to TMZ treatment. After treatment with the TMZ-Velcade regimen, approximately 10% to 15% clonogenic survival was seen in both U87v and U87MG DN p53, suggesting that delayed Velcade treatment abolishes the protective effect of mutant p53 against TMZ.

Proteasome Inhibition Sensitized GBM Neurosphere Lines to TMZ

Emerging literature suggests that GBMs consist of cells with differing potential in terms of self-renewal, differentiation, and proliferation. Within each tumor are minority populations that have potent tumorigenicity, referred to as cancer stem or progenitor cells. These populations are also extremely resistant to DNA damaging agents such as IR.[3] For the Velcade/TMZ treatment strategy to be viable in the clinical setting, it must be effective against these stem/progenitor cells.

To determine whether delayed Velcade treatment augmented TMZ activity against stem/progenitor cells, we tested this regimen against primary GBM neurospheres that exhibit characteristics associated with cancer stem cells including self-renewal and multipotency.[62] The neurosphere line BT69 (CD133+, Olig2+, GFAP+, Nestin+, epidermal growth factor receptor amplified), BT74 (CD133+, Olig2+, GFAP+, Nestin+, no epidermal growth factor receptor amplification), and BT78 (CD133+, Olig2+, GFAP-, Nestin+, no epidermal growth factor receptor amplification) exhibited significant resistance against TMZ, such that minimal cell death was seen at 500 μM TMZ treatment (*Fig. 19.4*). In contrast, 30% to 50% of the cells died in response to treatment with 100 μM TMZ in cell lines without stem cell characteristics (U87MG, U343MG, U343MG, and A172). In all three neurosphere cell lines, delayed treatment with 1 nM Velcade resulted in significant sensitization to TMZ, suggesting that Velcade sensitization of TMZ is efficacious against stem or progenitor tumor cells. Similar results were obtained using a previously described CD133− neurosphere line (BT75, *Fig. 19.4D*).[62]

Delayed Velcade Treatment Altered TMZ-Induced G2/M Arrest

To better understand the mechanism by which proteasome inhibition sensitizes GBM cells to TMZ, we analyzed the cell cycle distribution of U87MG cells after the various regimens by flow cytometry. As shown in *Figure 19.5*, treatment with 5 nM of Velcade for 24 hours did not significantly alter cell cycle distribution compared with vehicle (dimethyl sulfoxide)-treated cells. As previously reported,[51] TMZ-treated cells progressed through one complete cell cycle and then arrested at the G2/M junction of the second cell cycle (day two). This arrest persisted well beyond day five (data not shown). In contrast, delayed Velcade treatment after TMZ caused G2/M arrested in the first cell cycle (day 1). Moreover, instead of the prolonged G2/M arrest seen in TMZ treated cells, the combined TMZ/Velcade treated cells exhibited decreased G2/M population by day three. Associated with this decreased accumulation was an increase subG1 population. These results suggest either that the G2/M arrested cells were dying or that the cells progressed through the G2/M checkpoint without repair of DNA damage.

Delayed Velcade Treatment After TMZ Exposure Caused Aberrant Mitotic Arrest and Cytoplasmic Enlargement

To determine cell fate after treatment with TMZ and Velcade, we used live cell imaging to observe the U87MG stably expressed histone H2B-GFP (*Fig. 19.6*). We found that TMZ-treated cells underwent abnormal mitosis at a higher rate (54.6%) compared with vehicle (dimethyl sulfoxide)-

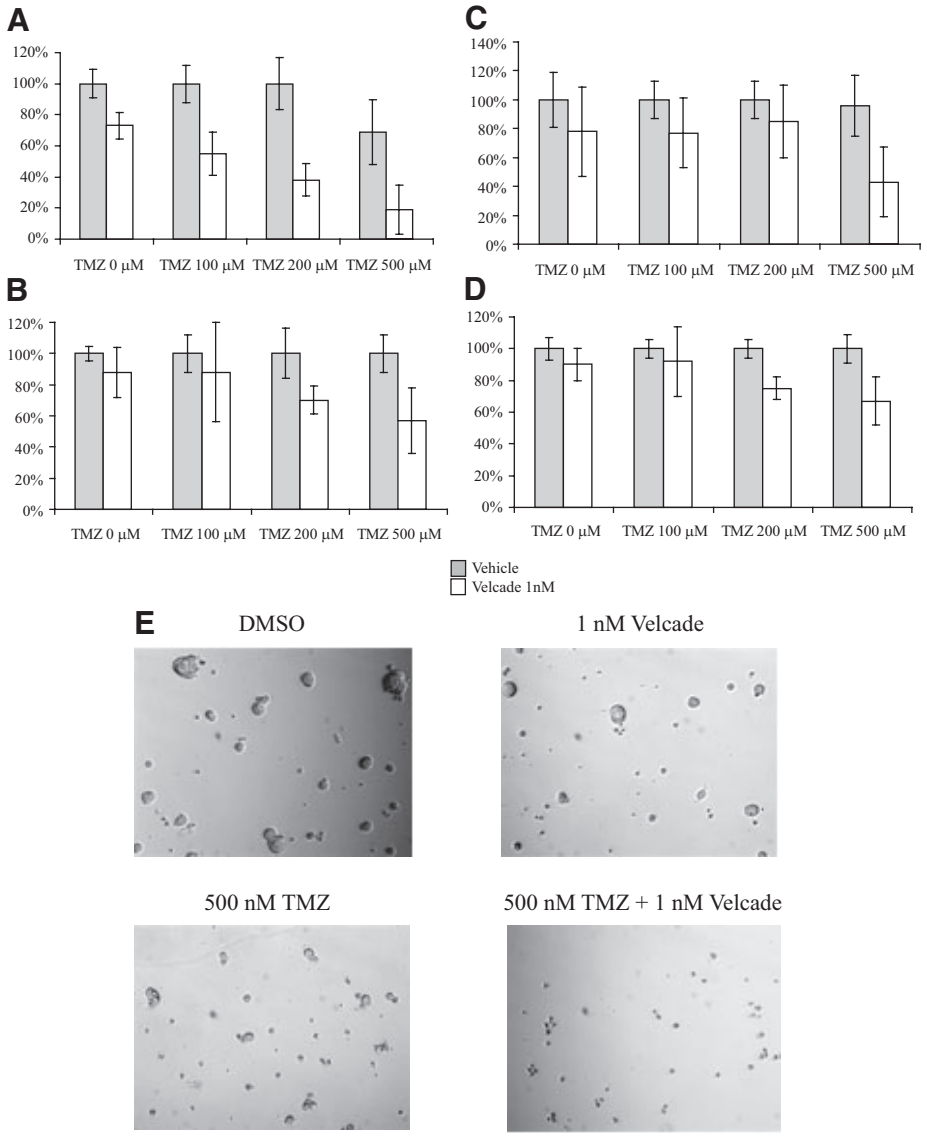


FIGURE 19.4. Proteasome inhibition sensitized glioblastoma multiforme (GBM) neurosphere cell lines to temozolomide (TMZ). GBM neurosphere lines expressing CD133[50] and Olig 2[29] were treated with various concentrations of TMZ for 24 hours followed by addition of Velcade (bortezomib) to a final concentration of 1 nM. Viability after treatment was measured after seven days using the MTT assay (see Methods). The percentage of survival after treatment was calculated by dividing the viability of treated cells relative to nontreated cells. The experiment was repeated twice. The results represent the average of triplicates. A representative experiment is shown for BT69 (**A**), BT74 (**B**), BT78 (**C**), and BT75 (**D**). **E,** Representative photomicrograph of BT74 after the various treatment regimens. DMSO, dimethyl sulfoxide.

treated cells (5.0%). Among all the abnormal mitoses, 72.2% exhibited chromosome missegregation and 27.8% exhibited cytokinesis failure. Delayed Velcade treatment after TMZ did not significantly alter the rate of abnormal mitoses.[37] However, it did alter the type of abnormal mitosis.[48] Specifically, delayed Velcade treatment after TMZ increased the number of cells with prolonged mitotic arrest relative to TMZ-treated cells (16.7% versus 0%, respectively). During the 72 hours of observation, we observed no mitotic death (n = 30). Furthermore, we found very few apoptotic cells. It appeared that most cells underwent prolonged cell cycle arrest or cellular senescence.

Examination of the cell phenotype at the end of the 72-hour incubation revealed that TMZ treatment caused a significant increase in the size of the cytoplasm without accompanying increase in the size of the nucleus. Delayed Velcade treatment after TMZ further increased the size of cytoplasm relative to that seen in TMZ-treated cells.

Proteasome Inhibition Sensitized the U87 Glioblastoma Cell Line to TMZ in an In Vivo Xenograft Model

Mice harboring established U87-Luciferase[43] xenograft tumors were divided into four groups. These groups were treated with TMZ, Velcade, TMZ + Velcade, and vehicle medium as described in Methods. Tumor growth derived based on serial measurements of bioluminescence revealed that Velcade treatment at the dose of 1 mg/kg (intravenously) caused an

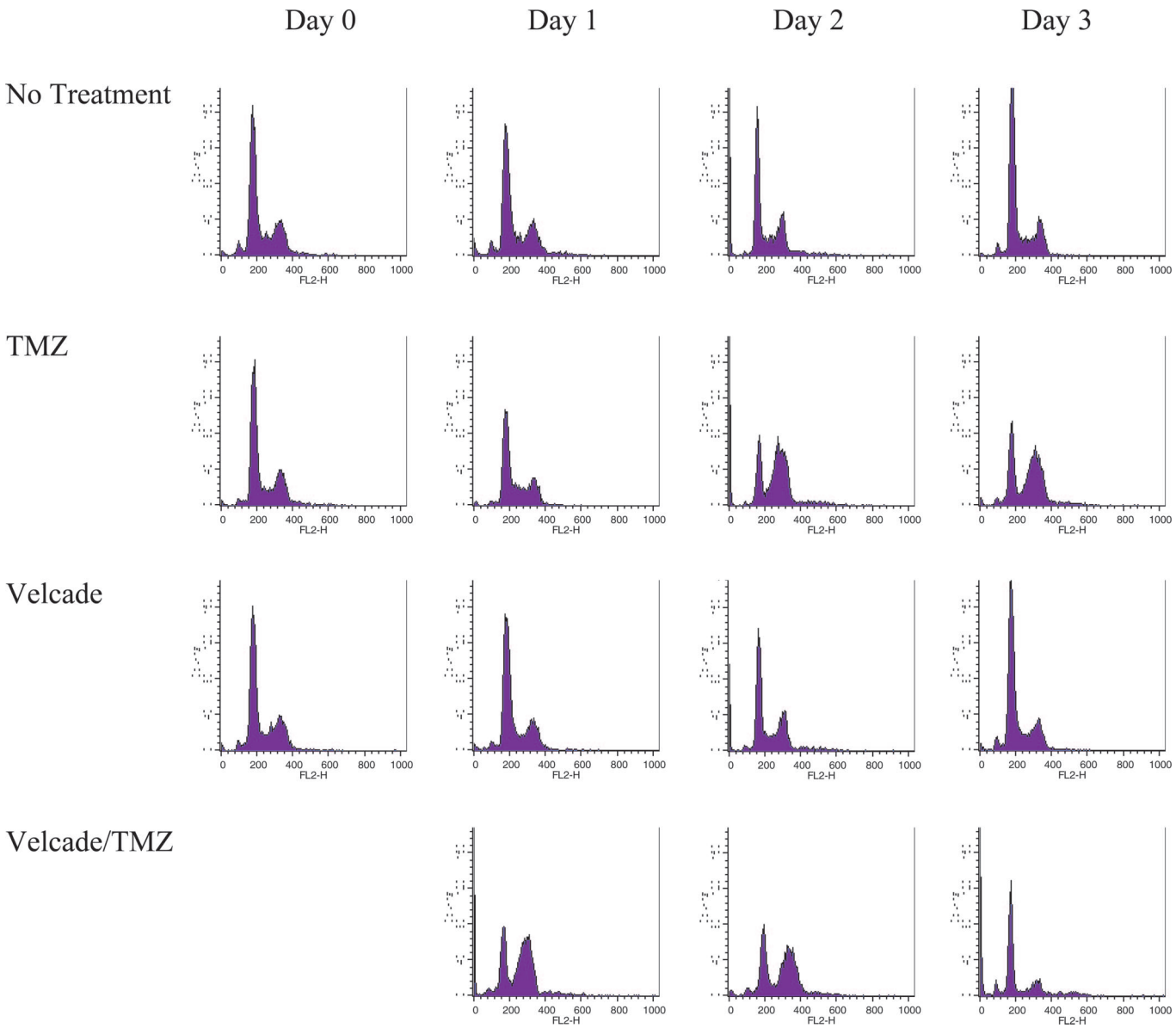


FIGURE 19.5. Velcade (bortezomib) pretreatment abolishes temozolomide (TMZ)-induced G2/M arrest. Flow cytometric analysis of U87MG cells treated with dimethyl sulfoxide, TMZ, Velcade (5 nM), or delayed Velcade (5 nM) treatment after TMZ. Cells were harvested and fixed at the indicated time and stained with propidium iodide (PI). Ten thousand cells were analyzed for each sample. DNA content measured by PI is represented on the x axis, the number of cells counted on the y axis. The experiments were repeated twice. A representative experiment is shown.

antineoplastic effect relative to the no treatment control. This result suggests that Velcade penetrated the blood-brain barrier of the tumor harboring mice. As anticipated, a three-day course of TMZ at 15 mg/kg/day orally significantly suppressed U87 growth in mice. This antineoplastic effect of TMZ was further augmented by the addition of Velcade (*Fig. 19.7*). There was no difference in mean body mass between groups nor was there other evidence of toxicity related to TMZ + Velcade-treated mice relative to the other groups (data not shown).

DISCUSSION

In this report, we used an siRNA-based screen to identify strategies that would augment the efficacy of radiation therapy and TMZ chemotherapy. Using this approach, we identified proteasome function as an important determinant for cellular sensitivity to IR and TMZ. We then searched for pharmacological inhibitors targeting this pathway. We found that independent proteasome inhibitors sensitized GBM cells to IR and TMZ. One of these inhibitors, Velcade,

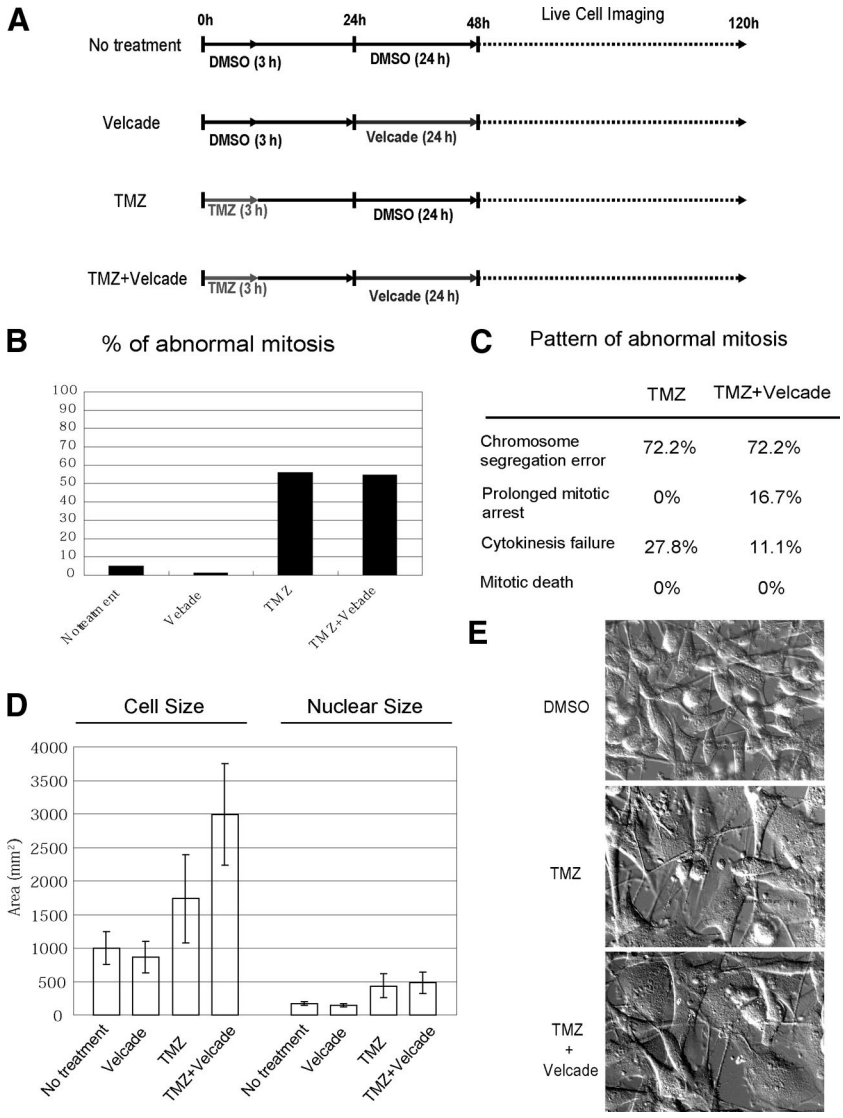


FIGURE 19.6. Velcade and temozolomide (TMZ)-treated cells exhibit increased mitotic arrest and expanded cytoplasm. **A,** U87MG H2B-GFP cells were treated with the various regimens as described and subjected to live cell imaging (see Methods for details). **B** and **C,** Increase in abnormal mitosis in the TMZ or TMZ+Velcade-treated cells. Live cell imaging was performed after drug treatment. Fluorescence and differential interference contrast images were taken every 15 minutes. At least 30 mitoses in each condition were observed, and abnormal mitosis was scored. Approximately 50 mitoses were scored. The percentage of cells undergoing aberrant mitosis is indicated in *Figure 19.6B*. Abnormal mitosis was further subclassified into segregation error, cytokinesis failure, mitotic arrest, and mitotic death[48] and shown in *Figure 19.6C*. **D** and **E,** Expanded cytoplasm in TMZ+Velcade-treated cells. Representative images of cell size at the 72-hour time point after the various treatment regimens (**B**). Cell and nuclear size at the 72-hour time point was measured using NIS element software ($n > 50$) (**E**).

is in clinical use for the treatment of multiple myeloma. Furthermore, this agent is currently undergoing clinical trial for the treatment of recurrent GBM. We reasoned that data obtained using Velcade may be translatable into the design of new clinical trials. We, therefore, characterized this agent in detail.

In accordance with previous reports, we found that Velcade exhibited tumoricidal activities at nanomolar concentrations.[39,53,60] At sublethal concentrations, Velcade enhanced the tumoricidal activity of IR and TMZ irrespective of the p53 status of the GBM cell lines. Importantly, we demonstrated this sensitizing effect in $CD133^+$ and $CD133^-$ neurosphere cell lines, suggesting the efficacy of TMZ-Velcade combination against cancer stem cells. Further, the sensitizing effect was observed in an in vivo xenograft model. Because IR and TMZ are rarely used in the treatment of recurrent GBMs, the benefit derived from the synergy between these agents would not be realized in this setting. Our data suggest that Velcade should be integrated into a combined regimen with IR and TMZ as an upfront therapy. Our data further suggest that Velcade should be administered after IR and TMZ to optimize its efficacy.

The tumoricidal effect of Velcade and TMZ appeared highly dependent on the order of addition. Although we initially postulated that pretreatment with Velcade may halt cell cycle progression and, thereby, inhibit the formation of TMZ-induced DNA strand breaks,[51] we find that the sublethal concentration of Velcade required for TMZ sensitization did not significantly alter cell cycle progression (*Fig. 19.5*). The mechanism underlying this dependency, therefore, remains unclear. Similar dependencies on the order of addition were observed when combining other DNA damaging agents with Velcade. Treatment of myeloma cells with doxorubicin fol-

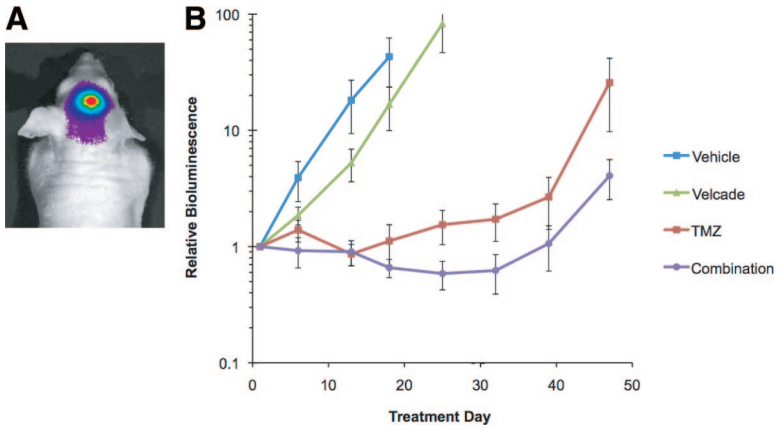


FIGURE 19.7. Proteasome inhibition sensitized the U87 glioblastoma cell line to temozolomide (TMZ) in an in vivo xenograft model. **A,** Bioluminescent image obtained from mice with successful xenograft uptake. Red indicates the highest density of photon flux and correlates with highest density of tumor cells. Purple indicates the lowest density of photon flux and tumor cells. **B,** Tumor growth curves were derived from serial measurements of bioluminescence as described in Methods. Data are mean values and standard error of the mean. Treatment groups are as indicated. A representative experiment is shown.

lowed by Velcade led to a stronger synergistic induction of cell death compared with treatment using the reverse sequence.[34] Interestingly, proteasome inhibition was shown to inhibit cellular response to DNA damage, including up-regulation of DNA repair genes.[32] Blunting a resistance mechanism, such as DNA repair up-regulation, that occurs specifically after DNA damage could potentially explain why delayed Velcade treatment after TMZ is more effective to pretreatment or simultaneous treatment with Velcade.

One factor that limits Velcade as therapy for glioblastoma lies in its inability to penetrate the intact blood-brain barrier.[40,44] However, several lines of evidence suggest that Velcade can cross a compromised blood-brain barrier. First, Velcade is shown to reduce the size of infarction in rat models of focal cerebral ischemia, suggesting CNS penetrance after ischemia-induced blood-brain barrier breakdown.[20] Second, the hydrophilicity and molecular weight of Velcade is highly comparable to that of the gadolinium-based contrast material routinely used in magnetic resonance imaging, such as gadodiamide.[45] Because neither agent is subjected to active transport mechanisms, the similarity in chemical properties and molecular size suggests that Velcade will perfuse the same region occupied by gadolinium contrast agents. Finally, the in vivo intracranial xenograft model used in this study suggests that Velcade alone or in combination with TMZ penetrates the CNS to exert antineoplastic effects.

The efficacy of Velcade in GBM therapy is, therefore, entirely dependent on the regional breakdown of the blood-brain barrier within tumor volume.[27] As such, we propose that quantitative assessment of blood-brain barrier breakdown be performed using dynamic computed tomography or magnetic resonance imaging sequences[55] as a part of patient selection. The combined TMZ/IR/Velcade therapy should be offered to those patients with significant blood-barrier breakdown within the tumor volume.

Alternative approaches to incorporate proteasome inhibition into an clinical trial include local convection or wafer delivery[28,59] or the select use of proteasome inhibitors that cross the blood-brain barrier.[57] However, these approaches require serious consideration of potential CNS toxicities. As Velcade fails to cross the normal blood-brain barrier in human patients, CNS toxicities have yet been reported in the treatment of multiple myeloma. However, genetic inactivation of proteasome function in the CNS is associated with the development of Parkinson's disease.[4] In this context, convection direct delivery of proteasome inhibitors or implant of proteasome inhibitor–coated wafers[28] should be approached with caution.

We showed that the sensitizing effect of Velcade is in part attributable to altered DNA damage check response at the G2/M junction and mitotic checkpoint. Of note, the mechanisms regulating these checkpoints are distinct.[19,56] It is likely that distinct proteins critical to these pathways are either directly or indirectly degraded by proteasome during normal cell physiology. Because the proteasome complex is involved in the degradation of a wide spectrum of proteins participating in distinct cellular processes, the effect of its inhibition is expected to be pleotropic. Understanding the specific mechanism of sensitization will, therefore, be challenging. However, such an understanding is required for rational development of future therapeutic strategies.

In summary, our results suggest that proteasome inhibition significantly augments the tumoricidal effect of IR and TMZ. Based on our study, we propose that selected GBM patients may benefit from the integration of Velcade into a regimen involving TMZ and IR as upfront therapy.

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Disclosure

The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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