

Aggravated Endoplasmic Reticulum Stress as a Basis for Enhanced Glioblastoma Cell Killing by Bortezomib in Combination with Celecoxib or Its Non-Coxib Analogue, 2,5-Dimethyl-Celecoxib

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Abstract

The proteasome inhibitor bortezomib (Velcade) is known to trigger endoplasmic reticulum (ER) stress via the accumulation of obsolete and damaged proteins. The selective cyclooxygenase-2 (COX-2) inhibitor celecoxib (Celebrex) causes ER stress through a different mechanism (i.e., by causing leakage of calcium from the ER into the cytosol). Each of these two mechanisms has been implicated in the anticancer effects of the respective drug. We therefore investigated whether the combination of these two drugs would lead to further increased ER stress and would enhance their antitumor efficacy. With the use of human glioblastoma cell lines, we show that this is indeed the case. When combined, bortezomib and celecoxib triggered elevated expression of the ER stress markers GRP78/BiP and CHOP/GADD153, caused activation of c-Jun NH₂-terminal kinase and ER stress-associated caspase-4, and greatly increased apoptotic cell death. Small interfering RNA-mediated knockdown of the protective ER chaperone GRP78/BiP further sensitized the tumor cells to killing by the drug combination. The contribution of celecoxib was independent of the inhibition of COX-2 because a non-coxib analogue of this drug, 2,5-dimethyl-celecoxib (DMC), faithfully and more potently mimicked these combination effects *in vitro* and *in vivo*. Taken together, our results show that combining bortezomib with celecoxib or DMC very potently triggers the ER stress response and results in greatly increased glioblastoma cytotoxicity. We propose that this novel drug combination should receive further evaluation as a potentially effective anticancer therapy. [Cancer Res 2008;68(3):843–51]

Introduction

The endoplasmic reticulum (ER) stress response (ESR) consists of a set of adaptive pathways that can be triggered by disparate perturbations of normal ER function, such as accumulation of unfolded proteins, lipid or glycolipid imbalances, or changes in the ionic conditions of the ER lumen (see refs. 1, 2 for reviews). The primary purpose of the ESR is to alleviate the stressful disturbance and restore proper ER homeostasis; however, in the case of intense or persistent ER stress, these pathways will trigger programmed

cell death/apoptosis. One of the central prosurvival regulators of the ESR is glucose-regulated protein 78 (GRP78/BiP), which has important roles in protein folding and assembly, in targeting misfolded protein for degradation, in ER Ca²⁺ binding, and in controlling the activation of transmembrane ER stress sensors (3). On the other hand, CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) and caspase-4 are critical executioners of the proapoptotic arm of the ESR (4, 5). In cases of intense or persistently high stress, the defensive functions (such as elevated levels of GRP78) are being overwhelmed, and the proapoptotic components (such as CHOP and caspase-4) will gain dominance and trigger cell death.

Bortezomib (Velcade, PS-341) has been approved for the treatment of multiple myeloma and is being investigated for the potential therapy of other tumors as well (6, 7). It functions as a selective inhibitor of the 26S proteasome, a multisubunit protein complex that is responsible for the degradation of ubiquitinated proteins, which include damaged and obsolete proteins that need to be deleted from the inventory of the cell (8). Recent studies have indicated that induction of cell death by bortezomib is a result of the accumulation of misfolded and otherwise damaged proteins, thereby leading to the activation of the unfolded protein response, which in turn leads to the activation of the ESR (9, 10). It is thought that, in the continuous presence of this drug, ever increasing levels of obsolete proteins are overwhelming the protective efforts of the ESR (e.g., increased levels of the chaperone GRP78) and trigger cell death via the stimulation of CHOP and other proapoptotic components of the ESR (11).

Celecoxib is a selective inhibitor of cyclooxygenase-2 (COX-2) and is widely prescribed under the trade name Celebrex for relief of symptoms of osteoarthritis and rheumatoid arthritis; it was also approved as an adjunct to the standard of care for patients with familial adenomatous polyposis. In the laboratory, celecoxib has shown potent anticancer activity in various animal tumor models, and it is suspected that this drug might be useful for the treatment of colorectal and possibly other types of cancer as well (12–14).

The underlying molecular mechanisms by which celecoxib exerts its antitumor effects are not entirely understood, in particular due to an increasing number of reports describing potent antiproliferative and proapoptotic effects of this drug in the absence of any apparent involvement of COX-2 (15–22). One of several lines of evidence excluding the potential contribution of COX-2 came from the use of 2,5-dimethyl-celecoxib (DMC), a close structural analogue of celecoxib that lacks the ability to inhibit COX-2 activity (23). Despite its inability to inhibit COX-2, DMC faithfully and more potently mimics all of the numerous antitumor effects

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in vitro and *in vivo* of celecoxib, including the reduction of neovascularization and the inhibition of experimental tumor growth in various animal tumor models (18, 24, 31).

Recent reports have established the ESR as a major mechanism by which celecoxib and DMC can trigger tumor cell death *in vitro* and *in vivo* (25, 32, 33). The initiating event seems to be the inhibition of the sarcoplasmic/ER calcium ATPase (SERCA), which is accomplished within seconds of adding celecoxib or DMC to cultured cells and which results in extensive leakage of calcium into the cytosol (25, 34, 35). As a consequence of this acute shift in subcellular calcium distribution, severe ER stress is triggered. In the continued presence of drug, the protective components of the ESR are unable to restore proper calcium homeostasis, and therefore, the proapoptotic arm of the ESR gains dominance and initiates tumor cell death (25, 32–37).

In view of the differential ESR-inducing insults generated by bortezomib and celecoxib/DMC, we set out to explore whether the combination of these stressors would potentiate the antitumor potency of these drugs. Our hypothesis was that the combination of two effective triggers of ER stress (i.e., disturbed calcium homeostasis and accumulation of obsolete proteins) would more effectively overwhelm the protective features of the ESR and therefore more potently stimulate tumor cell death. In this report, we show that this is indeed the case. The addition of bortezomib together with celecoxib or DMC to glioblastoma cells in culture more severely triggered the ESR and caused substantially more cell death than either drug alone. These events could also be shown in xenograft tumor tissues from drug-treated animals, indicating that these mechanisms have *in vivo* relevance. Taken together, our study establishes bortezomib and celecoxib/DMC as a novel drug combination that should be evaluated further as a potentially effective anticancer therapy.

Materials and Methods

Materials. Bortezomib was obtained from the pharmacy as 3.5 mg Velcade suspended in 3.5 mL saline (Millennium Pharmaceuticals). Celecoxib was obtained as capsules from the pharmacy or synthesized in our laboratory according to previously published procedures (38). DMC is a close structural analogue of celecoxib, where the 5-aryl moiety has been altered by replacing 4-methylphenyl with 2,5-dimethylphenyl (18, 27); this compound was synthesized in our laboratory according to previously published procedures (27). Celecoxib and DMC were dissolved in DMSO at 100 mmol/L (stock solution) and added to the cell culture medium in a manner that kept the final concentration of solvent below 0.1%. The COX-2-inhibitory activity of freshly synthesized celecoxib, and lack thereof in DMC, was confirmed *in vitro* with the use of purified COX-2 protein (see, for example, ref. 23).

Cell lines and culture conditions. All cells were propagated in DMEM (Cellgro) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator at 37°C and a 5% CO₂ atmosphere. Four human glioblastoma cell lines (LN229, U251, T98G, and U87MG) and one multiple myeloma cell line (RPMI/8226) were used. T98G, U87MG, and RPMI/8226 cells were obtained from the American Type Culture Collection. LN229 and U251 were obtained from Frank B. Furnari (Ludwig Institute of Cancer Research, La Jolla, CA). COX-2 expression levels and drug effects on prostaglandin production in these cells have been published elsewhere (17, 19).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed in 96-well plates with the use of 3.0×10^3 to 8.0×10^3 cells per well as described in detail elsewhere (26).

Cell death ELISA. Cells were plated in 96-well plates in quadruplicates at 1,000 cells/mL (100 μ L/well). The next day, they were treated with drugs

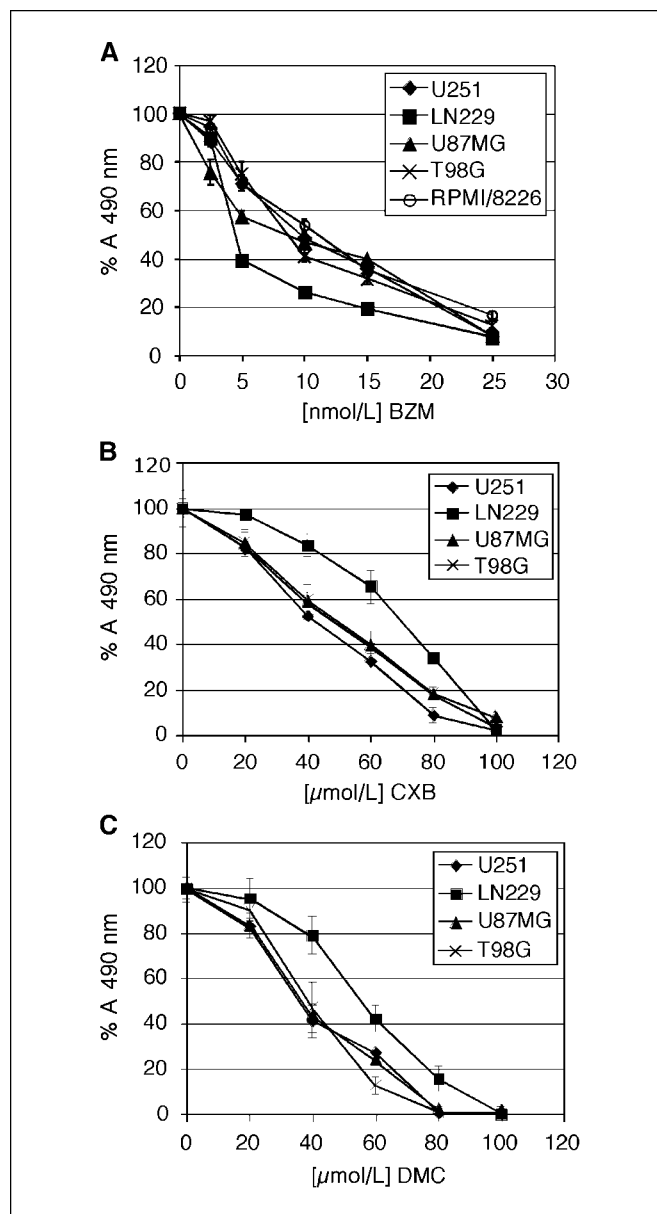


Figure 1. Bortezomib, celecoxib, and DMC reduce glioblastoma cell survival. Cell growth and survival of various glioblastoma cell lines was determined by MTT assay after 48 h of culture in the presence of increasing concentrations of bortezomib (BZM; A), celecoxib (CXB; B), or DMC (C). For comparison purposes to the known anti-multiple myeloma effects of bortezomib, the RPMI/8226 multiple myeloma cell line was included in A.

for 24 h and analyzed for the presence of histone-complexed DNA fragments with the use of a commercially available ELISA kit (Roche Diagnostics) according to the manufacturer's instructions. The kit was used in a manner as to specifically quantitate apoptosis rather than necrosis.

Immunoblots and immunohistochemical staining. Total cell lysates were prepared and analyzed by Western blot analysis as described earlier (26). Immunohistochemical analysis of protein expression in tumor tissues was performed with the use of the Vectastain avidin-biotin complex method kit (Vector Laboratories) as described previously (25). The primary antibodies were purchased from Cell Signaling Technology or Santa Cruz Biotechnology, Inc. and used according to the manufacturers' recommendations. All immunoblots and stainings were repeated at least once to confirm the results.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of tumor tissue. Apoptosis in tumor sections was measured quantitatively with the use of the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. All components for this procedure were from the ApopTag *In Situ* Apoptosis Detection kit (Chemicon), which was used according to the manufacturer's instructions. The percentage of TUNEL-positive cells for each tumor section was determined from 10 random photomicrographs taken at $\times 200$ magnification.

Transfections and colony formation assay. The different small interfering RNAs (siRNA) were synthesized at the microchemical core laboratory of the University of Southern California/Norris Comprehensive Cancer Center; their sequences are described in ref. 25. Transfections of cells with these siRNAs and subsequent analysis of cell survival by colony formation assay have been described in detail elsewhere (25).

Drug treatment of nude mice. Four- to 6-week-old male athymic *nu/nu* mice were obtained from Harlan and implanted s.c. with 5×10^5 U87 glioblastoma cells. Once tumors of $\sim 300 \text{ mm}^3$ had developed, the animals received drug treatments. Velcade was given as a single dose via tail vein injection. DMC was given twice daily (one half of the daily dose every 12 h) via direct administration into the stomach with a stainless steel ball-head feeding needle (Popper and Sons, Inc.). After a total of 50 h, the animals were sacrificed and tumors were collected for analysis. In all experiments, the animals were closely monitored with regards to body weight, food consumption, and clinical signs of toxicity; no differences between non-drug-treated control animals and drug-treated animals were detected.

Results

Glioblastoma multiforme represents a particularly difficult-to-treat type of cancer with dismal prognosis. Because more effective therapies are urgently needed, we chose various human glioblastoma cell lines as a model to investigate combination effects of our selected drugs. To establish the concentration of each drug that would result in 50% inhibition of cell growth (IC_{50}), we first treated each cell line individually with either bortezomib, celecoxib, or the non-coxib celecoxib analogue DMC. The resulting IC_{50} after 48 h of treatment with bortezomib was $\sim 10 \text{ nmol/L}$ for the U251, U87MG, and T98G glioblastoma cell lines and slightly below 5 nmol/L for the LN229 glioblastoma cell line (Fig. 1A). Because bortezomib was developed for multiple myeloma therapy and is highly cytotoxic in such tumor cell lines, we also determined its IC_{50} in a representative multiple myeloma cell line, RPMI/8226, for comparison purposes. As shown in Fig. 1A, and as expected, RPMI/8226 cells exhibited high sensitivity toward bortezomib; however, this cell type was not more sensitive than the glioblastoma cell lines. This finding established that glioblastoma cells were exquisitely sensitive to bortezomib, which supported our rationale for investigating this drug as a potential glioblastoma therapy.

We also established the IC_{50} for celecoxib and DMC in all four glioblastoma cell lines (Fig. 1A and B). Celecoxib displayed an IC_{50} of $\sim 50 \text{ } \mu\text{mol/L}$ in U251, U87MG, and T98G cells, whereas DMC was somewhat more potent with an IC_{50} of slightly below $40 \text{ } \mu\text{mol/L}$. The LN229 cell line (which exhibited the greatest sensitivity to bortezomib; Fig. 1A) was overall slightly less sensitive to either celecoxib or DMC (Fig. 1A and B).

To determine the ability of bortezomib, celecoxib, and DMC to induce the ESR in glioblastoma cells, we treated U251 cells with increasing concentrations of each drug and analyzed various indicators of ER stress. As shown in Fig. 2A, bortezomib caused increased expression of GRP78 and CHOP, indicating that the ESR was triggered. In addition, this drug also stimulated the activation of the ER stress-associated procaspase-4, as indicated by the appearance of the cleaved (i.e., activated) form of this enzyme.

To verify that bortezomib, at the concentrations we used, exerted its established function (i.e., inhibition of the proteasome), we also investigated the accumulation of polyubiquitinated proteins. As shown in Fig. 2A, the induction of ER stress markers coincided with the appearance of highly elevated levels of polyubiquitinated proteins, indicating that inhibition of the proteasome correlated with the induction of ER stress.

The same targets were also investigated after treatment of cells with either celecoxib or DMC. In all our experiments, celecoxib and DMC consistently generated the same outcome, except that DMC was slightly more potent [for this reason, and also because the induction of ER stress by celecoxib has been reported earlier (25, 32, 33), we will focus primarily on the results obtained with DMC]. As shown in Fig. 2B, DMC treatment resulted in strong induction of GRP78 and CHOP and activation of caspase-4,

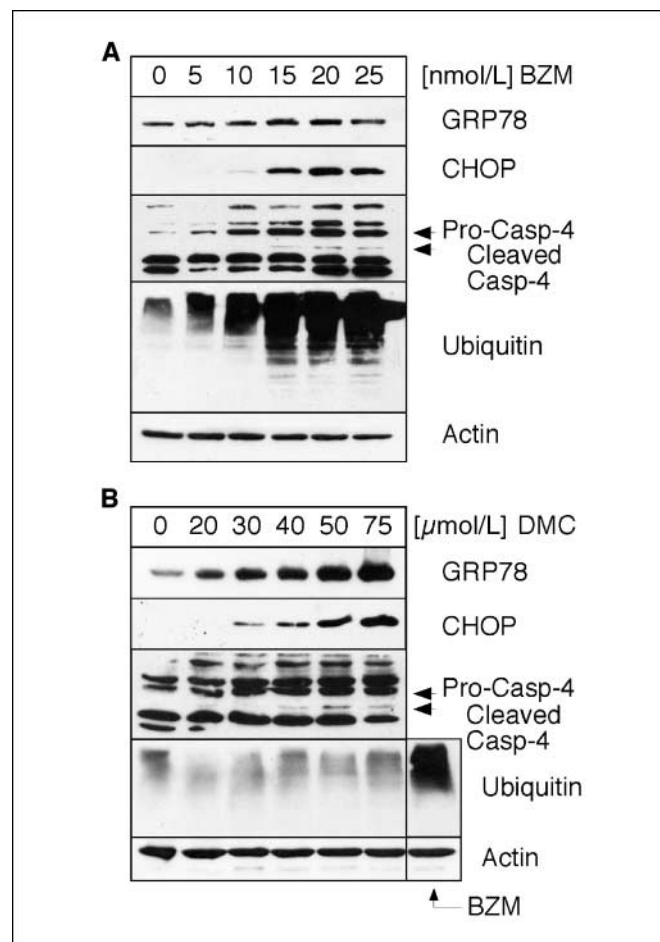


Figure 2. DMC and bortezomib induce indicators of ER stress. U251 glioblastoma cells were cultured for 24 h in the presence of increasing concentrations of bortezomib (A) or DMC (B). Total cell lysates were analyzed by Western blot with specific antibodies to ubiquitin, GRP78, CHOP, and caspase-4. Actin was used as a loading control. Procaspase-4 (*Pro-Casp-4*) denotes the inactive caspase-4 proenzyme, whereas cleaved caspase-4 (*Casp-4*) is indicative of the activated form of the enzyme. For comparison purposes, cells treated with 10 nmol/L bortezomib were analyzed side by side to DMC-treated cells in B, indicating that the amount of ubiquitination is much more prevalent in bortezomib-treated cells than in DMC-treated cells. Note that in the caspase-4 blots several nonspecific background bands were observed (consistent with similar observations in the literature); the specific bands were identified with the use of various controls (data not shown) and comparison with the literature.

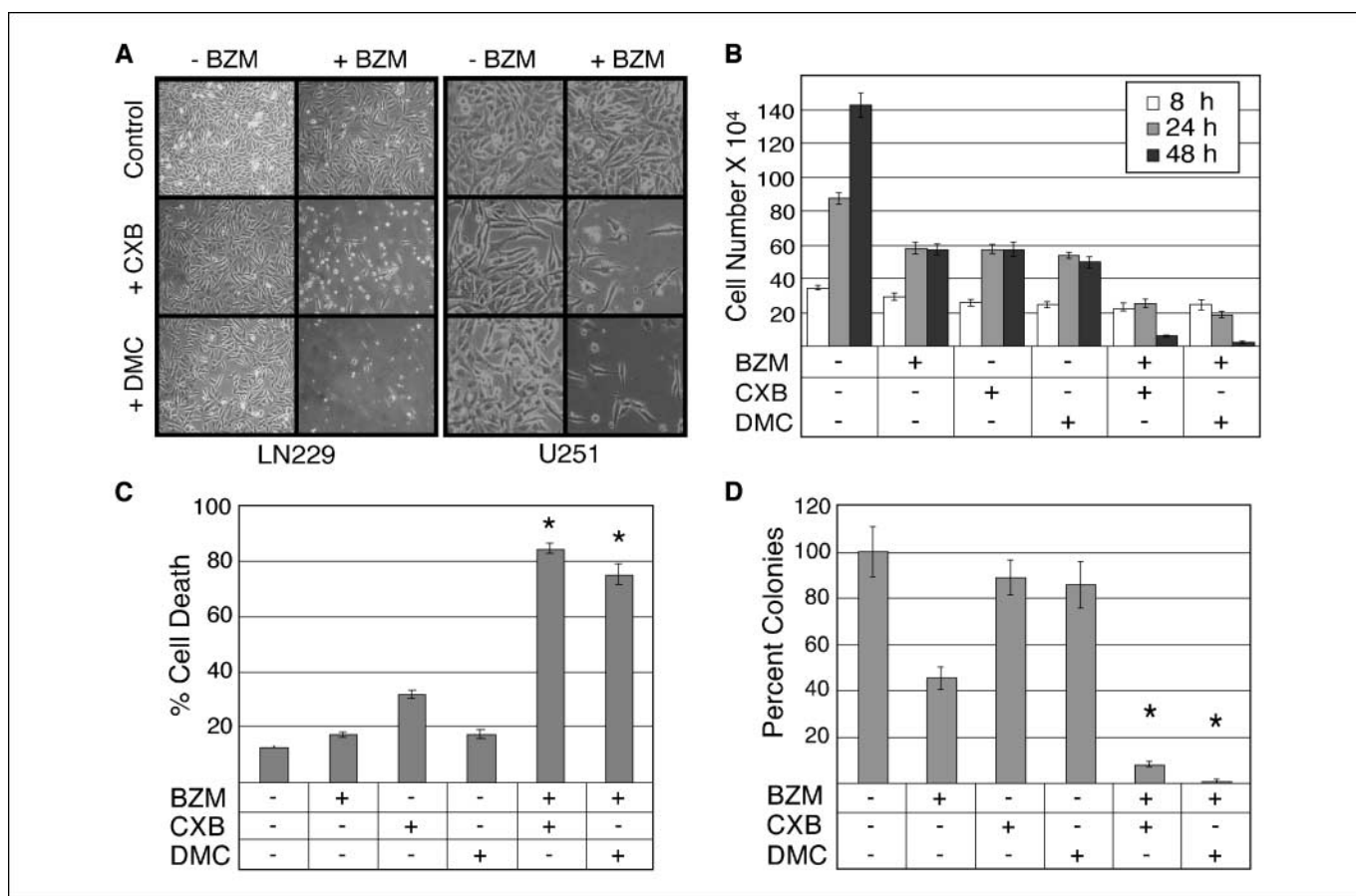


Figure 3. Celecoxib and DMC enhance growth inhibition and cell death by bortezomib. Glioblastoma cell lines were treated with bortezomib, celecoxib, or DMC, alone and in combination. *A*, photomicrographs depicting the effects of combined drug treatment in LN229 and U251 cells after 48 h of drug treatment. Representative sections are shown. *B*, quantitative analysis of combination drug effects. LN229 cells were treated with drugs as above for 8, 24, and 48 h. Cell viability was determined by trypan blue exclusion assay. The assays were performed with triplicate samples, and results are representative of three independent experiments. *Columns*, mean number of viable cells under each condition; *bars*, SD. *C*, U87MG cells were cultured for 24 h in the presence of drugs as indicated, and the extent of cell death was determined by cell death ELISA. *Columns*, mean percent ($n = 4$); *bars*, SD. *D*, LN229 cells were treated with drugs for 48 h and the number of long-term surviving cells that were able to spawn a colony was determined 2 wk thereafter (colony formation assay). *Columns*, mean percentage of surviving cells from triplicate experiments; *bars*, SD. The number of colonies obtained from non-drug-treated controls was set at 100%. In *A* to *D*, the following drug concentrations were used: LN229: 5 nmol/L bortezomib, 60 μ mol/L celecoxib, 40 μ mol/L DMC; U251: 10 nmol/L bortezomib, 50 μ mol/L celecoxib, 30 μ mol/L DMC; U87MG: 5 nmol/L bortezomib, 50 μ mol/L celecoxib, 35 μ mol/L DMC. *, $P < 0.001$, difference between individual drug treatments and combination drug treatments was statistically highly significant.

indicating that this drug triggered ER stress. However, this drug did not cause substantial accumulation of polyubiquitinated proteins, consistent with the expectation that its mechanism of action was different from that of bortezomib.

We next examined the effects of combination drug treatments on glioblastoma cell growth and survival. For this purpose, we combined bortezomib with either celecoxib or DMC at concentrations that represented approximate IC_{50} values so that potentially enhancing effects would become apparent. Figure 3*A* depicts a visual display of cell number and morphology, whereas Fig. 3*B* presents the quantified outcome. Figure 3*A* reveals that individual drug treatment resulted in a smaller increase in cell number and the presence of fewer mitotic figures; in contrast, combination drug treatment resulted in noticeable cell loss and apparent cell death. These visual impressions were complemented by counting the number of viable cells over the course of drug treatment (at 8, 24, and 48 h). As displayed in Fig. 3*B*, single-drug treatment allowed initial cell proliferation (i.e., increase in cell numbers between 8 and 24 h) and then exerted presumed

cytostatic effects (i.e., the overall number of cells did not change between 24 and 48 h). In contrast, when the drugs were applied in combination, there was no increase in cell numbers at the 24-h time point and substantial loss of viable cells between 24 and 48 h, indicating potent cytotoxic effects.

The extent of drug-induced cell death and survival was further investigated by cell death ELISA, which quantitates the amount of apoptosis in the entire culture, and by colony formation assays, which determine the number of individual cells able to survive drug treatment long-term and spawn a colony of clonal descendants. Figure 3*C* shows that treatment of glioblastoma cells with individual drugs caused a small increase in apoptotic cell death, whereas the combined treatment with bortezomib and celecoxib or DMC resulted in greatly increased cell death. In colony formation assays, 5 nmol/L bortezomib reduced the number of emerging colonies by $\sim 50\%$ (Fig. 3*D*). The chosen concentrations of celecoxib and DMC by themselves exerted only minor inhibitory effects (reduction of colony number by approximately 10–15%). In contrast, when cells were treated with bortezomib and celecoxib

or DMC in combination, colony survival was greatly reduced by >90% and 97%, respectively.

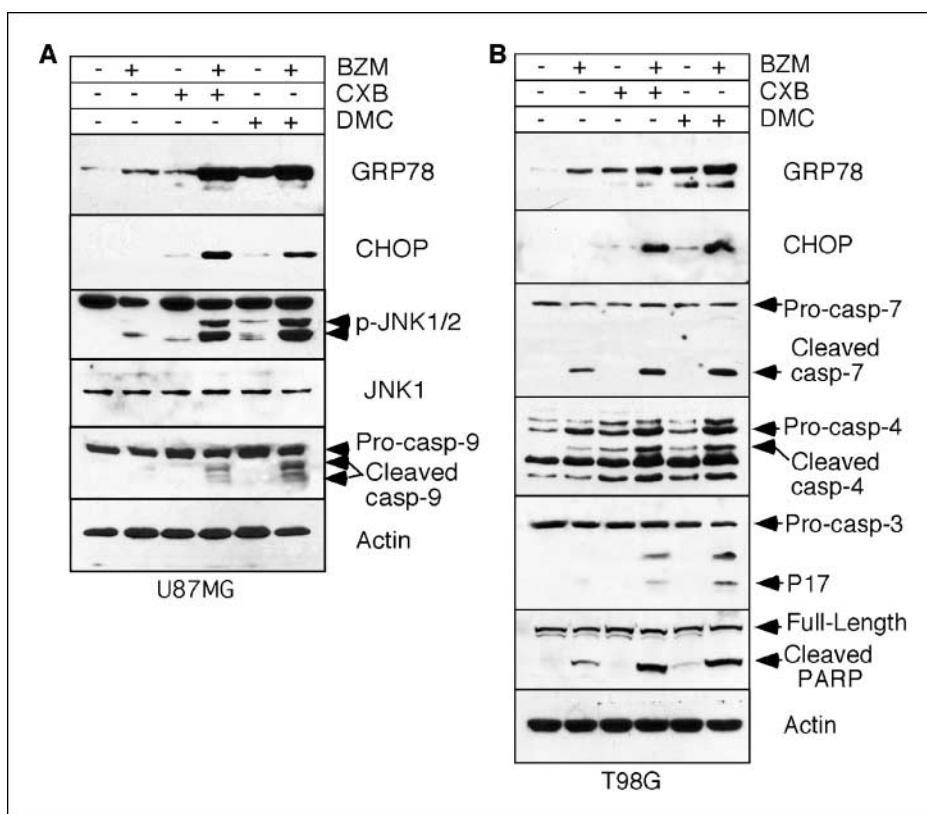
Each assay used in Fig. 3 was applied to several different glioblastoma cell lines and varying concentrations of drug combinations were used (data not shown). In all cases, very similar outcomes were achieved, clearly indicating that the combination of these drugs results in greatly increased cytotoxicity and substantially reduced cell survival compared with treatment with each drug individually. Furthermore, we calculated the combination index (CI) from conventional MTT assays where increasing concentrations of each drug were combined (data not shown) and obtained a CI <1, revealing that the drug combination effects were synergistic.

We next investigated the potential contribution of the ESR to the above-presented combination drug effects. U87MG and T98G cells were treated with the same drug combinations as above, and various components of the ESR system and cell death machinery were analyzed. As shown in Fig. 4, individual drug treatments resulted in increased expression of GRP78, and combination drug treatments increased expression of this protein further. Levels of the proapoptotic CHOP protein were weakly increased by single-drug treatments but were more strongly elevated by combination treatments. The activity of c-Jun NH₂-terminal kinase (JNK), a critical proapoptotic component of the ESR, was investigated with antibodies specifically recognizing the phosphorylated (i.e., active) form of this kinase. We found that combination drug treatments, but not individual drug treatments, resulted in greatly increased JNK activity (Fig. 4A). Taken together, these results indicate that bortezomib, when combined with either celecoxib or DMC, caused stronger ESR induction than either drug alone. Similar results were also obtained with the use of LN229 and U251 cells (data not shown).

To investigate the role of apoptosis, the activity of various caspases in response to drug treatment was determined. Activation of each caspase can be detected by the appearance of a proteolytically cleaved, smaller molecular weight form of the protein. As shown in Fig. 4, the ER-associated caspase-4 and caspase-7 were more strongly activated by combination drug treatments than by individual drug treatments. In addition, caspase-9, a member of the intrinsic apoptotic pathway, was activated by the drug combinations but was much less affected by individual drugs. As well, the executioner caspase-3 was substantially more activated by the drug combinations than by individual drugs. Finally, poly(ADP-ribose) polymerase-1 (PARP-1), one of the main substrates of activated caspase pathways and a well-established indicator of apoptotic cell death, was more effectively cleaved in response to combination treatments than by individual drug treatments (Fig. 4). Thus, these results reveal a close correlation of ER stress induction, activation of ER stress-associated caspases, and additional apoptotic events that indicate the dominance of proapoptotic mechanisms in response to combination drug treatment.

To establish whether drug-induced ER stress and cellular apoptosis were only correlative or were causally related, we specifically reduced the expression of the ESR component GRP78. If drug-induced cell death was controlled by the ESR, we would expect that reduced levels of GRP78, which functions as a major protective component of the ESR, would lead to further increased cell death. U251 cells were transfected with siRNA against GRP78; as a control, cells were transfected with siRNA against a target not present in mammalian cells [i.e., green fluorescent protein (GFP)]. Both siGRP78-transfected and siGFP-transfected cells were treated with bortezomib together with celecoxib or DMC, and the number

Figure 4. Celecoxib and DMC enhance up-regulation of indicators of ER stress and apoptosis by bortezomib. U87MG (A) and T98G (B) cells were cultured in the presence of 10 nmol/L bortezomib, 50 μmol/L celecoxib, or 35 μmol/L DMC individually or in combination as indicated for 24 h. Total cell lysates were analyzed by Western blot with specific antibodies to GRP78, CHOP, caspase-3 (*casp-3*), caspase-4, caspase-7 (*casp-7*), caspase-9 (*casp-9*), PARP, and JNK, as indicated. Actin was used as a loading control. Procaspase denotes the full-length (inactive) caspase proenzymes, whereas cleaved caspases represent the activated forms of these enzymes. The activity of JNK1 and JNK2 was determined with the use of an antibody that specifically recognizes JNK phosphorylated on Thr¹⁸²/Tyr¹⁸⁵ (*p-JNK1/2*). Equal amounts of JNK1 were confirmed with an antibody that reacts with all JNK forms present.



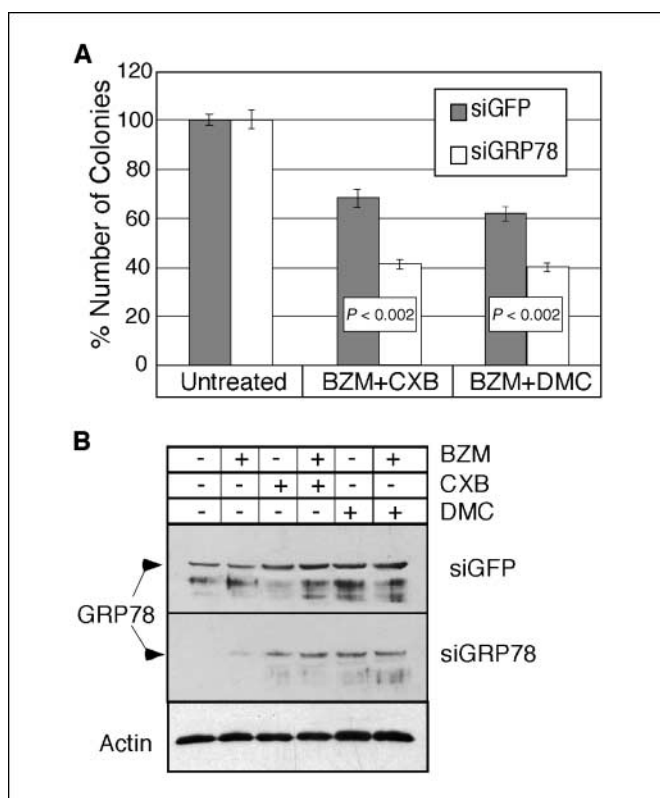


Figure 5. Knockdown of GRP78 enhances cell killing by combination drug treatment. U251 cells were transiently transfected with siRNA directed at GRP78 (*siGRP78*). As a control, a siRNA targeted at GFP (*siGFP*) was used. **A**, 72 h after transfection, parallel cultures were treated with 5 nmol/L bortezomib combined with either 25 μ mol/L celecoxib or 15 μ mol/L DMC. In parallel, transfected control cultures received no drug treatment or treatment with solvent (DMSO) alone. After 48 h, the drugs were removed and the fraction of surviving cells was determined by colony-forming assays over the course of 12 to 14 d. Shown is the percentage of surviving cells able to spawn a colony (where the number of colonies under non-drug-treated conditions was set to 100%). The reduction in colony numbers in drug-treated *siGRP78*-transfected versus drug-treated *siGFP*-transfected cells was statistically significant ($P < 0.002$). **B**, to verify the effectiveness and specificity of the transfected siRNAs, Western blot analysis of GRP78 expression was performed from *siGFP*-transfected and *siGRP78*-transfected cells treated with the respective drugs in parallel. Both of these blots were processed and developed simultaneously and therefore can be directly compared side by side. Although the down-regulation of GRP78 by its siRNA was not 100% effective, the levels of this protein nonetheless were consistently lowered in each condition compared with the matching control cells transfected with *siGFP*. Of note, overall lower concentrations of each drug were used in this experiment to enable the detection of further enhanced cell death by siRNA pretreatment.

of surviving cells was determined by colony formation assay. As shown in Fig. 5A, cell survival was significantly ($P < 0.002$) decreased in cells harboring reduced levels of GRP78; cell survival after drug treatment was 63% to 68% in cells transfected with *siGFP* but was decreased to 40% to 42% in cells transfected with *siGRP78*. Down-regulation of GRP78 expression by *siGRP78* was confirmed by Western blot analysis; however, whereas the presence of *siGRP78* reduced basal levels of GRP78 below the detection limit, the siRNA could not completely block the induction of GRP78 by drug treatment, as lower levels of induced GRP78 protein could still be detected (Fig. 5B). Nonetheless, under all conditions, the overall amount of GRP78 protein was lower in *siGRP78*-transfected cells than in *siGFP*-transfected cells. Concurrently, *siGRP78*-transfected cells exhibited significantly increased chemosensitivity, indicating

that the ESR played a causal role in triggering cell death induced by bortezomib in combination with celecoxib or DMC.

Finally, we determined whether the above-described *in vitro* events would also take place *in vivo*. U87MG cells were implanted s.c. into nude mice, and after sizable tumors had formed, the animals remained untreated or were treated with drugs. Celecoxib was not included in this experiment because previous studies had already shown that this drug potently stimulated the ESR *in vivo* (25, 32); furthermore, all known experiments that compared DMC to celecoxib have shown that both drugs reliably achieve the same tumor-suppressive outcome *in vitro* and *in vivo*, except that DMC consistently displays somewhat greater apoptosis-inducing potency (18, 25–27, 30). Therefore, we decided on DMC as the more potent drug of choice for the *in vivo* combination experiments with bortezomib.

We had shown previously that DMC by itself quite potently triggers ER stress in tumor tissue *in vivo* (25), and this effect begins to appear at dosages of ≥ 10 mg/kg. Considering these earlier results, we chose 7.5 mg/kg as a potentially useful dosage for this combination experiment; we reasoned that suboptimal dosages of DMC would allow the combination effects to emerge. Tumor-bearing animals were treated with DMC or bortezomib alone or in combination for 2 days (a relatively short treatment period was chosen because we wanted to focus on the early mechanisms that initiate cell death rather than the later conditions that dominate when the tumor cells are dying or already are dead). Tumor tissue was analyzed for the expression of CHOP (as a marker of ER stress) and stained by TUNEL (to visualize the extent of apoptotic cell death).

We found that DMC, at this very low dosage, did not cause much elevated CHOP expression nor did it substantially increase TUNEL staining (Fig. 6A). Bortezomib treatment by itself resulted in a larger fraction of CHOP-positive cells, concomitant with increased TUNEL staining. In comparison, when bortezomib was given together with DMC, there was a very strong induction of CHOP, which could be detected in every single cell, and an even greater increase in the number of TUNEL-positive cells (Fig. 6A). When the number of TUNEL-positive cells in the various treatment groups was quantitated, it became apparent that combination treatment resulted in significantly more cell death than individual drug treatment (Fig. 6B); that is, cell death in response to treatment with bortezomib plus DMC was 4.9- and 3.0-fold higher than in animals treated with DMC or bortezomib alone, respectively. Thus, combination drug treatment resulted in substantially higher levels of ER stress and a greater amount of cell death than individual drug treatments and established that aggravated ER stress and enhanced glioblastoma cell killing could be achieved *in vivo* as well.

Discussion

The critical contribution of the ESR to tumor cell growth and survival has begun to be recognized only very recently (1–3). On one hand, this system supports tumor cell survival under adverse conditions, such as hypoxia, low glucose levels, or chemotherapeutic intervention, and recent evidence indicates that indeed many tumor cells exhibit increased survival due to chronic ER stress (39). On the other hand, if the stressful insult becomes too severe and cannot be adequately neutralized, the ESR switches from its protective function to its proapoptotic properties and triggers cell death. Although the crucial relevance of these mechanisms to tumor growth and survival is being recognized,

very little is known with regards to their potential exploitation for purposes of tumor therapy. We therefore investigated the possibility that aggravating ER stress via the simultaneous application of two different ESR-triggering drugs would lead to increased tumor cell death.

For our experiments, we chose three different drugs that were known to be able to trigger the ESR and cause tumor cell death. Bortezomib triggers ESR secondary to the accumulation of misfolded and otherwise damaged proteins (9, 10). Celecoxib and its non-coxib analogue DMC stimulate ESR due to the inhibition of SERCA and the subsequent leakage of calcium from the ER into the cytosol (30, 34). Although not a Food and Drug Administration-approved drug, DMC was included in this study because this compound generally exhibits greater antitumor potency than celecoxib *in vitro* and *in vivo* (18, 25, 30). In addition, because it lacks COX-2-inhibitory function, the use of DMC permits the evaluation of a potential role for COX-2 in the processes investigated (23). This latter aspect is important in view of the known life-threatening cardiovascular risks that have emerged with the long-term use of coxibs and are associated with the selective inhibition of COX-2 (40). In this regard, the absence of COX-2-inhibitory potential in DMC might turn out to be advantageous, as it is conceivable that the antitumor results might be achieved with less of the coxib-associated side effects (23).

We found that, when applied as single-drug treatment, bortezomib, celecoxib, and DMC dose dependently reduced the growth and survival of various glioblastoma cell lines (Fig. 1). Consistent with earlier results in several different tumor cell types

(18, 25, 26), DMC was noticeably more potent than celecoxib. With bortezomib, we found that all glioblastoma cell lines were highly sensitive to this drug with IC_{50} s of below 5 nmol/L (LN229 cell line), 8 nmol/L (T98G), and 10 nmol/L (U87MG and U251). This exquisite sensitivity was somewhat surprising in light of earlier results indicating a wide range of sensitivities and even relative bortezomib resistance of glioblastoma cell lines. For instance, Yin et al. (41) reported IC_{50} s for T98G, U87MG, and U343 cells of 9, 80, and 800 nmol/L, and Styczynski et al. (42) determined these values in T98G and U373 cells as 29 and 48 nmol/L, respectively. The reason for these discrepancies between the same cell lines is unclear (all studies used MTT assays); however, it is noted that, when we included a representative multiple myeloma cell line to allow direct, side-by-side comparisons of drug sensitivities, we found that this cell line responded in line with the glioblastoma cell lines tested (IC_{50} = 12 nmol/L; Fig. 1A). Thus, in contrast to indications by earlier reports, bortezomib, which has been approved for the treatment of multiple myeloma, did not seem to be less cytotoxic to glioblastoma cells.

When combined with either celecoxib or DMC, bortezomib displayed greatly increased cytotoxicity (Fig. 3), and these enhanced combination effects correlated closely with aggravated ER stress (Fig. 4). ER stress was indicated by the increased expression or activity of the following ESR components: (a) GRP78/BiP, an ER chaperone and critical component of the protective arm of the ESR (3); (b) CHOP/GADD153, a transcription factor and proapoptotic constituent of the ESR (1); (c) JNK, a stress-activated kinase that plays a proapoptotic role during the ESR and is involved in

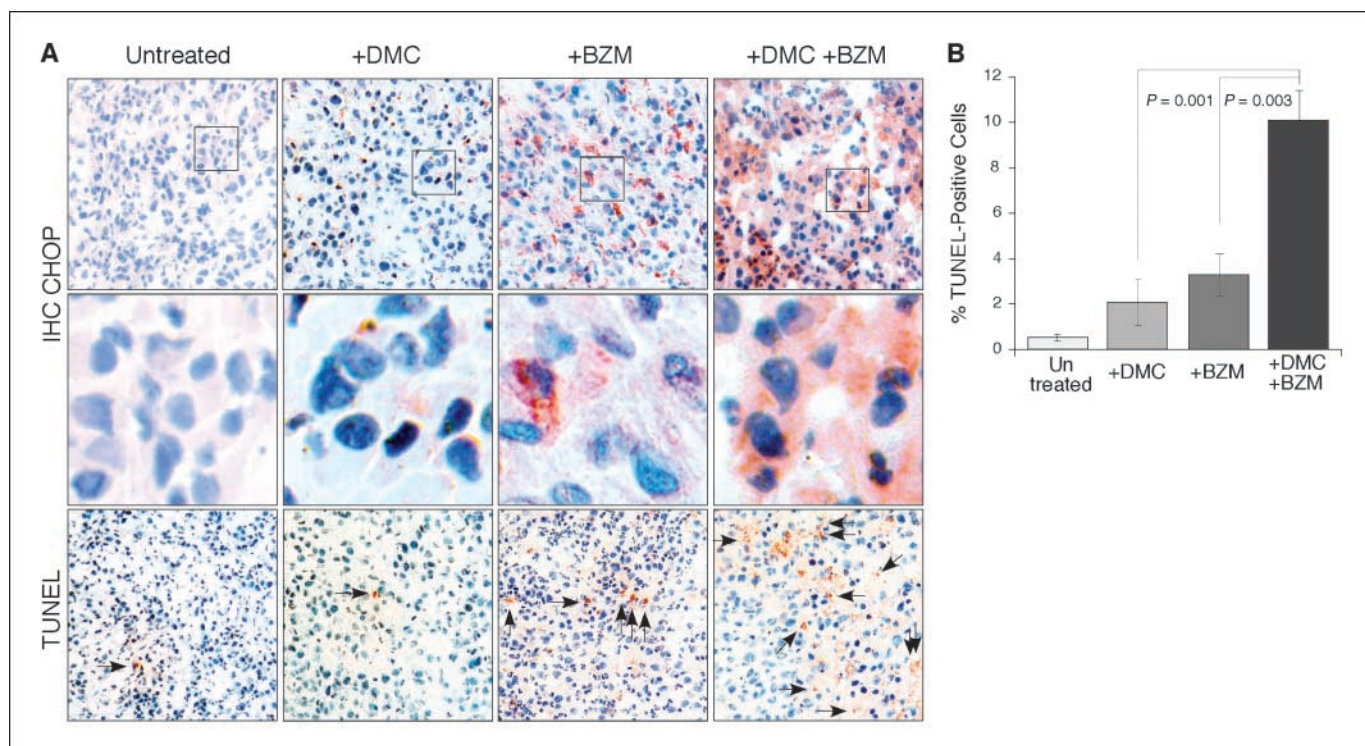


Figure 6. DMC enhances the effects of bortezomib on ER stress and apoptosis *in vivo*. Tumor-bearing mice were treated with 1 mg/kg bortezomib and 7.5 mg/kg DMC individually or in combination or remained untreated. Fifty hours later, the animals were sacrificed and the tumors were analyzed by immunohistochemical (IHC) staining for CHOP (ER stress indicator) or by TUNEL (apoptosis indicator). **A**, top, tumor tissue stained with CHOP antibodies; middle, enlarged areas (small rectangles) of the same sections; bottom, TUNEL staining. Arrows, a few select TUNEL-positive cells. **B**, the percentage of TUNEL-positive cells (reddish brown stain) was determined in 10 randomly chosen microscopic fields from each treatment group. Columns, mean; bars, SD. Statistically significant differences in the extent of tumor cell death between individual and combination drug treatments are indicated in the chart.

mediating cell death induced by bortezomib (41, 43); (d) caspase-4, which is localized to the ER and is involved in ER stress-induced apoptosis (4, 44, 45); and (e) caspase-7, also an ER stress-associated protein, whose activity is tightly regulated via the interaction with GRP78 (46–48). We found that all of the above components were more potently stimulated by combination drug treatments than by individual drug treatments, clearly indicating the presence of severe ER stress. In addition, activation of the intrinsic apoptotic pathway became evident by the activation of caspase-9 and caspase-3 and by cleavage of PARP-1 (Fig. 5), which is consistent with the current model of ER stress-induced apoptosis (1).

The causal relationship of drug-induced ER stress and ensuing cell death was established via the knockdown of GRP78 expression with siRNA. This type of approach has been used before to establish the protective function of this ER chaperone during treatment with celecoxib alone (32). In the present study, we show that reduced levels of GRP78 protein significantly decreased the survival of glioblastoma cells after combination treatment, showing that when this protective ESR component is impaired, the cells become even more sensitive to combination drug-induced cell killing. In view of the established model of the ESR (1–3), we surmise that, due to the loss of protection by GRP78, the proapoptotic components of the ESR are able to intensify their dominance and trigger more effective cell death.

In the past, studies that had used celecoxib and DMC received the criticism that drug effects *in vitro* generally required much higher concentrations than those that can be achieved *in vivo* in the serum of patients or animals; it was therefore concluded that the observed *in vitro* mechanisms might not be reflective of the events taking place *in vivo* (49). Recently, however, several studies verified that some of the high-concentration *in vitro* effects can also be detected in xenograft tumor tissues *in vivo*. For instance, the induction of ER stress by celecoxib and DMC *in vitro*, as indicated by increased expression of GRP78 and

CHOP, requires minimum concentrations of each drug that are well above 10 $\mu\text{mol/L}$ (25, 32, 33). In comparison, increased expression of GRP78 and CHOP can also be detected in tumor tissues from animals treated with celecoxib or DMC (25, 32), although the average concentrations of these drugs *in vivo* are <10 $\mu\text{mol/L}$ in serum and <0.3 $\mu\text{mol/L}$ in tumor tissue (18, 25). Although a solution to the conundrum of differential drug concentration requirements between *in vitro* and *in vivo* conditions has yet to be provided, these types of experiments nonetheless strongly caution against the tendency to minimize results that were obtained with the use of moderately high micromolar concentrations of celecoxib and DMC *in vitro*. Our present results shown in Fig. 6 further support this view, as they clearly show the *in vivo* relevance of our study.

In summary, we have provided evidence that the combination of two unrelated drugs, bortezomib and celecoxib/DMC, exerts greatly increased cytotoxicity on glioblastoma cells. Each drug by itself is able to induce ER stress by a different mechanism, and their combination further aggravates ER stress to the point where the protective components of this system are being overwhelmed and the proapoptotic constituents gain dominance and effect tumor cell death. Because the ESR already is chronically activated in some tumor cells, there might be reduced competence to accommodate additional insults that specifically target the ESR and therefore might provide a therapeutic window for those drugs aimed at this cellular mechanism.

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