Synergistic Killing of Glioblastoma Stem-like Cells by Bortezomib and HDAC Inhibitors

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Abstract. Background: The malignant brain tumour glioblastoma is a devastating disease that remains a therapeutic challenge. Materials and Methods: Effects of combinations of the US Food and Drug Administration (FDA) approved proteasome inhibitor bortezomib and the histone deacetylase (HDAC) inhibitors vorinostat, valproic acid and sodium phenylbutyrate were studied on primary glioblastoma stem cell lines and conventional glioblastoma cell lines. Cell survival, proliferation and death were analyzed by fluorometric microculture cytotoxicity assay (FMCA), propidium iodide labeling and flow cytometry, and cell cloning through limiting dilution and live-cell bright-field microscopy. Results: Bortezomib and the HDAC inhibitors showed synergistic cell killing at clinically relevant drug concentrations, while the conventional cell lines cultured in serum-containing medium were relatively resistant to the same treatments. Conclusion: These findings of synergistic glioblastoma stem cell killing by bortezomib and three different FDA-approved HDAC inhibitors confirm and expand previous observations on co-operative effects between these classes of drugs.

Glioblastoma is the most common type of primary brain tumour in adults. Following the introduction of combined treatment of temozolomide and radiotherapy, there has been a significant increase in median survival (1). However, median survival is still dismal, only slightly exceeding one year. Thus, there is an urgent need for new and improved treatment strategies. It is hypothesized that various types of cancers are maintained and propagated by small subpopulations of cells, known as cancer stem cells (also called tumour-initiating cells or tumour-propagating cells) (2, 3). This subpopulation of cells is postulated to possess the capacity for self-renewal, the ability to initiate and sustain tumour growth, and the potential to differentiate into various cancer cell types present in the tumour. It has been argued that treatment modalities that effectively eliminate cancer stem cell populations have the potential to be effective or even curative against human cancers (2). In glioblastoma, cancer stem cells have been isolated based on the expression of cell surface markers, such as CD133 (3, 4), CD15 (5) and integrin α6 (6), and also by their ability to efficiently efflux certain dyes (7). Recently, methods to cultivate glioblastoma stem cells in serum-free neural stem cell medium on non-adhesive (3, 4, 8, 9) and laminin-coated (10) surfaces, have been described. Glioblastoma cells cultured under these conditions also seem to retain the genotypic and phenotypic features of the original tumour cells better than cells cultured in traditional serum-containing media (9). Since these in vitro-cultured stem cells mimic important features of glioblastoma stem cells in vivo, drugs and drug combinations that show activity in these cultures may also be effective against human glioblastoma in the clinic. Recently, various molecularly targeted therapies have been developed. However, these therapies have not yet improved the survival of patients with glioblastoma. Two non-mutually exclusive reasons for this failure could be that glioblastoma stem cells are not efficiently targeted and that the complexity and redundancy of molecular signaling pathways driving glioblastoma growth makes single pathway-targeting ineffective. The proteasome is involved in the degradation of a variety of cellular proteins. The antitumour effects of proteasome inhibitors are well-documented, but the exact mechanisms that underlie their preferential killing of tumour cells is not entirely clear (11). Bortezomib (PS-341; market name, Velcade) is a boronic-acid peptide that inhibits the 20S subunit of the 26S proteasome (12). Bortezomib has beneficial clinical antitumour activities and a mild toxicity profile. In a recent phase I clinical trial for recurrent malignant glioma, bortezomib had some clinical activity as a monotherapy (13).
Histone deacetylases (HDACs) catalyze the de-acetylation of lysines on histones and other proteins. Recent studies have demonstrated that the activity of HDAC inhibitors is not limited to histone modification, but also includes modifications of a wide range of non-histone proteins, including transcription factors, signal transduction mediators, chaperones, and microtubule components (14-16). Vorinostat (suberoylanilide hydroxamic acid, SAHA; market name, Zolinza) is an HDAC class I and II inhibitor. A recent phase II trial in patients with recurrent glioblastoma showed that vorinostat was well-tolerated and had some clinical activity as a monotherapy (17). Valproic acid and sodium phenylbutyrate are also inhibitors of HDACs (18-22). Valproic acid is a widely used antiepileptic drug, whereas sodium phenylbutyrate has been used to treat urea cycle disorders. Sodium phenylbutyrate has shown activity in patients with malignant gliomas, with modest side-effects (23, 24). There is accumulating pre-clinical evidence that bortezomib and vorinostat have co-operative antitumor effects in esophageal cancer (25), non-small cell lung cancer (26), hepatoma (27), mantle cell lymphoma (28), uterine cervical cancer (29), colon cancer (30) and T-cell lymphoma (31, 32). In primary cultures of glioblastomas, Yu et al. found co-operative effects between bortezomib and different experimental HDAC inhibitors (33). These results encouraged the current study, where we evaluated the effects of combinations of bortezomib and different US Food and Drug Administration (FDA)-approved HDAC inhibitors at clinically relevant concentrations on both glioma stem cell cultures and conventionally cultured glioma cell lines.

Materials and Methods

Cell lines. U251-MG and GL15 cell lines were derived from human glioblastoma and were generously provided by Monica Nistér (Karolinska Institute, Stockholm, Sweden) and Jean-Sébastien Guillamo (Centre Hospitalier Universitaire Côte de Nacre, Caen, France), respectively. The R11 cell line was established from a human glioblastoma under so-called neural stem cell culture conditions by Beier and co-workers (4). The TB101 cell line was established from a human glioblastoma by a similar protocol in our laboratory (approved by the Ethics Committee of Umeå University Hospital). U251-MG and GL15 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin. R11 and TB101 cells were cultured in neural stem cell medium on laminin-coated surfaces, as described by Pollard et al. (10).

Drugs. Bortezomib, vorinostat and sodium phenylbutyrate were generously provided by Janssen-Cilag (Millenium Pharmaceuticals, Inc., Cambridge, MA, USA), Merck Sharp & Dohme (Stockholm, Sweden), and Fyrklövern Scandinavia AB (Mönsterås, Sweden), respectively. Stock solutions were prepared by dissolving the drugs at 500 µM (bortezomib) or 1 M (sodium phenylbutyrate) in phosphate-buffered saline, or at 500 µM in dimethyl sulphoxide (vorinostat). The stock solutions were stored at −20°C until use. Valproic acid (Ergenyl, Sanofi Aventis, Stockholm, Sweden) was obtained from the hospital pharmacy at NUS, Umeå, Sweden. Valproic acid was kept in dried form at room temperature. A stock solution of valproic acid 400 mg/ml was prepared immediately before use.

Cytotoxicity assay. Cells were seeded in 96-well microtiter plates and cultured under the conditions described above. The day after seeding, drugs were added individually, or in combination, at the indicated concentrations, and the cells were incubated for an additional 48 h. Relative cell numbers were determined indirectly using the fluorometric microculture cytotoxicity assay (FMCA) (34). Control cells were handled in the same way, except for the absence of drugs in the cell culture medium.

Propidium iodide staining and flow cytometry. The proportion of dead cells was determined by labeling the cells with propidium iodide and performance of flow cytometric analysis, as previously described (35).

Limiting dilution analysis. To determine the colony-formation frequency, TB101 cells were serially diluted and seeded on laminin-coated microtiter plates. Cell densities ranged from an average of 1 to 1,000 cells/well. The day after seeding, cells were treated for 0, 6, 12 or 24 h with a combination of 5 nM of bortezomib and 2.5 µM of vorinostat. Thereafter, the wells were washed and fresh medium was added. The medium was subsequently changed once per week. Four weeks after the drug treatment, plates were visually inspected using an inverted microscope, and the colony-containing wells were counted. The frequency of colony-forming cells was calculated assuming a Poisson distribution.

Time-lapse microscopy. Approximately 400,000 cells were seeded per well in optic 12-well glass plates (MatTek, Ashland, MA, USA). The cells were either treated or not treated with 5 nM bortezomib and 2.5 µM vorinostat for 48 h. Bright-field images were acquired every 30 min using a LSM 710 microscope equipped with a 20×/0.8 apochromat objective controlled by the Zen 2010 software (Carl Zeiss Microscopy, GmbH, Jena, Germany).

Statistical analysis. Drug interaction effects were analyzed by two-way ANOVA. The null hypothesis was that the drug interaction was additive. If the interaction term from the ANOVA analysis was found to be significant, we concluded that the drugs displayed an overall synergy at the given concentrations. p-Values for the interaction term are presented. Because the ANOVA fits a linear model, we restricted our analyses to the linear range of the data. Therefore, in the analyses of bortezomib in combination with vorinostat for the TB101 and R11 cell lines (Figure 1A and B), the highest concentrations of each drug were excluded from ANOVA analyses.

Results

Glioblastoma stem cell cultures are sensitive to combinations of bortezomib and HDAC inhibitors. In a combinatorial drug screen, we observed that combinations of the proteasome inhibitor bortezomib and the HDAC inhibitor vorinostat had remarkable effects on the morphology and survival of glioblastoma stem cell cultures. To further investigate these effects, we analyzed the survival of glioblastoma cells following 48 h of incubation with different concentrations of
the two drugs, alone and in combination (Figure 1A-D). The glioblastoma stem cell lines TB101 and R11 were highly sensitive to combinations of bortezomib and vorinostat (Figure 1A and B). Bortezomib and vorinostat interacted synergistically, as determined by a two-way ANOVA, in both TB101 \((p<10^{-8})\) and R11 \((p<10^{-12})\) cells. The two conventional glioblastoma cell lines, GL15 and U-251 MG, which were cultured in FBS-containing medium, were more
resistant to the two drugs and to drug combinations. U-251 MG cells were synergistically sensitive to high concentrations of the drugs \( (p<0.001) \), whereas GL15 cells were insensitive to all tested drug concentrations (Figure 1C and D).

Next, we investigated whether the synergism between bortezomib and vorinostat was drug-specific or if bortezomib also interacted synergistically with other clinically approved HDAC inhibitors. Bortezomib was cross-titrated with valproic acid, and phenylbutyrate, and the survival of TB101 cells was analyzed (Figure 1E and F). Clearly, bortezomib interacted synergistically with both valproic acid \( (p=10^{-5}) \) and phenylbutyrate \( (p<0.001) \). To confirm the effects of the drugs on cell viability, propidium iodide uptake was analyzed by flow cytometry. TB101 and R11 cells were treated with 5 nM bortezomib and 2.5 μM vorinostat, and propidium iodide labeling was analyzed at different time points (Figure 2). Cells that had not been treated with the drugs had a viability of 80-90%, as defined by propidium iodide exclusion. In contrast, cells treated with drug combinations had a decrease in viability over time. After 48 h of exposure, only 10-20% of the drug-treated cells were viable.

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<table>
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<th>Time of treatment (hours)</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>24</th>
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<td>0.14</td>
<td>0.02</td>
<td>0.007</td>
<td>0.0005</td>
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aColony-forming efficiency was determined by limiting dilution analysis.

Colony-forming ability is a defining feature of cancer stem cells. To determine the effect of combining proteasome and HDAC inhibitors on the colony-forming ability of TB101 cells, limiting dilution analyses were performed (Table I). After as little as 6 h of treatment with bortezomib and vorinostat, the colony-forming efficiency of the TB101 cells was reduced from 0.14 for untreated cells to 0.02. After 24 h of treatment, the colony-forming efficiency was reduced even further, reaching 0.0005. In summary, one out of seven untreated cells formed a new colony, whereas only 1 of 2,000 cells, that had been treated with the drug combination for 24 h, were able to form a new colony.

Combination of bortezomib and vorinostat induces rapid morphological changes indicative of apoptosis. To obtain further insight into the kinetics and mechanisms by which glioblastoma cells were killed by the drug combination, the morphology of TB101 cells was followed by time-lapse microscopy (Figure 3; Supplementary movie, can be found online at: http://umu.diva-portal.org/smash/record.jsf?pid=diva2:536054). Nine hours of exposure to the drug combination induced morphological changes; the cells rounded up, detached from the substrate and exhibited extensive membrane blebbing and nuclear condensation, the latter two phenomena being indicative of apoptotic cell death.

### Discussion

The development of targeted agents has not yet resulted in any major improvement in the therapeutic outcome for patients with glioblastoma. In the present study, we evaluated possible cooperative effects of FDA-approved drugs that target proteasome and HDAC functions. We found that while either type of drug alone was only modestly toxic, combinations of bortezomib and HDAC inhibitors were highly toxic to the glioblastoma stem cell cultures, TB101 and R11, whereas the conventional cell lines were only modestly sensitive \( (U251-MG) \), or completely resistant \( (GL15) \). This confirms and extends previous findings \( (33) \). The differential drug effects among our glioblastoma cell cultures may suggest that glioblastoma stem cells are specifically sensitive to combinations of proteasome and HDAC inhibitors, while the majority of tumour cells (the tumour bulk), as represented by the conventional cell lines, is less sensitive. However, to confirm this hypothesis appropriate in vivo tumour
experiments need to be performed. The induced changes in cell morphology were indicative of apoptotic cell death. This is consistent with the findings of Yu et al. (33), who showed that combinations of proteasome and HDAC inhibitors induced apoptosis in established and short-term glioblastoma cell lines. Genetic evidence indicates that proteasome function is important for HDAC inhibitor-induced apoptosis (36). In addition, vorinostat inhibits aggresome formation, which could enhance the toxic effects of the bortezomib-induced accumulation of misfolded proteins (14). The relationships between our findings and those of Yu et al. (33) and other possible mechanisms involved, warrant further investigation. To effectively eradicate glioblastoma, it may be necessary to target glioblastoma stem cells (2). Although the frequency and exact nature of the putative glioblastoma stem cells is not firmly established, we decided to use glioblastoma stem cell cultures in our study. The glioblastoma stem cell cultures used were established and propagated on laminin in neural stem cell medium. These conditions favour the propagation of glioblastoma stem cells (10). Furthermore, glioblastoma cells cultured under these conditions retain the genotypic and phenotypic characteristics of the original tumour cells (9, 10). In contrast, conventional cell lines, such as GL15 and U-251 MG, when cultured in traditional serum-containing cell culture medium and maintained through a large number of passages, gradually lose the phenotype and the genotype of the original tumour cells (9, 37). This phenomenon could contribute to the relative resistance of GL15 and U-251 MG to bortezomib and vorinostat. Strikingly, the glioblastoma stem cell cultures (TB101 and R11) were highly sensitive to combinations of bortezomib and HDAC inhibitors, and the ability to form colonies was significantly reduced, while U-251 MG cells exhibited intermediary sensitivity and GL15 cells appeared to be resistant. The drugs used in the present study are all FDA approved, and the drug concentrations used are clinically achievable. For the four drugs used here, plasma concentrations in treated patients have been reported to be approximately 100 nM for bortezomib (38), in the micromolar range for vorinostat (39, 40), slightly below 1 mM for valproic acid (41), and in the millimolar range for sodium phenylbutyrate (23, 24). At these clinically relevant concentrations, the drug combinations were highly toxic to the glioblastoma stem cell cultures. During the preparation of this manuscript, results from a small clinical trial of heavily pre-treated patients with glioblastoma at disease recurrence, treated with a combination of bortezomib and vorinostat, were published (42). This study failed to show any clinical benefit of bortezomib and vorinostat for this patient group. The reason for the discrepancy between the pre-clinical findings and the lack of clinical benefit is probably complex. For example, both Yu et al. (33) and our group used treatment-naïve cells, while all of the patients in the clinical study were pre-treated with at least temozolomide, and more than half of the patients had also been treated with chemotherapy or bevacizumab, at recurrence (42). Although not stated in the article, a majority, if not all, of the patients had probably also undergone radiotherapy, which is standard as part
of first-line treatment. This could have a major impact on both cellular signaling and tumour microenvironment, and affect the response to subsequent therapy. In addition, it is possible that whereas glioblastoma stem cell populations, as represented by TB 101 and R11, may be sensitive to the treatment, the recurring tumour bulk, as represented by U251-MG and GL 15, may not be (43, 44). If so, combined proteasome and HDAC inhibitor treatment could be an interesting option for evaluation in patients with no or minimal residual disease (for example after surgery), where a recurrence may be dependent on the recruitment of glioblastoma stem cells. Alternatively, it would be desirable to combine the herein described anti-glioblastoma stem cell treatment with other drugs or strategies that target the tumour bulk. Thus, we propose that combinations of bortezomib and HDAC inhibitors that target glioblastoma stem cells could be of value in subpopulations of patients with glioblastoma with no or minimal residual disease, or in combination with other treatment modalities that target the bulk of tumour non-cancer stem cells.

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References


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