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SHORT COMMUNICATION

Bortezomib Downregulates MGMT Expression in T98G Glioblastoma Cells

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Abstract The efficacy of treatment for glioblastoma multiforme is currently limited by the development of resistance, particularly, but not exclusively, due to the expression of the DNA repair enzyme O6-methylguanine methyltransferase (MGMT) in a significant proportion of astrocytic tumors. MGMT is post-translationally regulated by the 26S proteasome, a multi-subunit organelle responsible for degradation of misfolded cellular proteins. The boronic acid dipeptide bortezomib is the first and only proteasome inhibitor in clinical use so far, and has been reported as a strategy to restrict growth and promote apoptosis of glioblastoma cells. In this study we investigated the effect of bortezomib on MGMT expression in T98G cells, looking for an effect on the nuclear factor kappa B (NFκB) pathway, which is a major player in MGMT regulation and is also under tight control by the ubiquitin–proteasome system. Administration of bortezomib led to a significant reduction of T98G cell viability and induction of DNA fragmentation. These effects coincided with reduced expression of MGMT transcript levels, and a decrease in cellular amount and IκBα-mediated, proteasomal activity-dependent nuclear translocation of NFκB. In addition, bortezomib-induced phosphorylation of the translation initiation factor 2alpha (eIF2α) was in parallel with translational repression of MGMT. Taken together, these results suggest a novel role for bortezomib as a potent MGMT inhibitor and support its ongoing testing as a chemosensitizer in glioblastoma.

Keywords MGMT · Bortezomib · NFκB · eIF2α · Glioblastoma · T98G

Introduction

Glioblastoma multiform represents the most common malignant primary brain tumor, with aggressive biological behavior and adverse prognosis (Preusser et al. 2011). Progress in understanding of the molecular pathogenesis of the disease and mechanisms of resistance to DNA alkylation damage induced by treatment with temozolomide has revealed a central role for the DNA repair protein O6-methylguanine methyltransferase (MGMT) (Christmann et al. 2011). The mechanism of action of MGMT involves the removal of alkyl groups from DNA by transferring them to an internal Cys-145 residue. As the S-alkylcysteine is not converted back to cysteine, the protein can only act once (suicide enzyme) and the resulting alkylated MGMT molecule is rapidly recognized by a ubiquitin ligase targeting the protein for proteolysis (Xu-Welliver and Pegg 2002).

In addition to MGMT, the ubiquitin–proteasome system is responsible for degradation of a plethora of cellular proteins involved in regulation of divergent processes such as proliferation, cell cycle, and apoptosis (Adams 2003). A key transcription factor whose nuclear translocation is orchestrated by the proteasome is nuclear factor kappa B (NFκB) (Lin et al. 2010). Notably, NFκB is also involved in transcriptional regulation of MGMT through direct interaction with two putative NFκB binding sites within the MGMT

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promoter region (Lavon et al. 2007). Bortezomib is currently the only proteasome inhibitor in clinical use, and it has been shown to cause cell growth arrest and apoptosis in glioblastoma through various mechanisms, including upregulation of cell cycle arrest and proapoptotic proteins (cyclin B1, p21, p27, Bmf, TRAIL DR5), enhancement of TRAIL-mediated apoptosis, downregulation of cell cycle progression and antiapoptotic proteins (CDK2, CDK4, E2F4, Bcl-2, Bcl-xl), and activated JNK signaling (Yin et al. 2005; Tianhu et al. 2010; Unterkircher et al. 2011; Seol 2011). In detail, bortezomib was found to cause accumulation of cyclin B1, p21 and p27 proteins which was associated with G2/M arrest. In addition, bortezomib induces attenuation of CDK2, CDK4, and E2F4 protein levels consistent with cells exiting the cell cycle. Furthermore, both mRNA and protein levels of Bcl-2 and Bcl-xl are significantly reduced after treatment with bortezomib, while the latter also markedly stimulates phosphorylation of JNK and c-Jun, which was partially reversed by a JNK inhibitor blocking bortezomib-induced cell death (Yin et al. 2005). A role of the JNK-Bmf pathway in mediating the apoptotic effects of bortezomib was suggested, based on evidence of Bmf protein and transcript levels upregulation by bortezomib which was blunted after inhibition of JNK phosphorylation (Tianhu et al. 2010). Also, bortezomib displays a synergistic apoptotic action when combined with TRAIL, which emanates from bortezomib-mediated tBid accumulation linking the extrinsic to the intrinsic apoptosis pathway and leading to enhanced Bax activation and mitochondria-driven cell death in glioma cells. At the TRAIL DISC, bortezomib concomitantly increases TRAIL receptor R2 (DR5) surface expression and cFLIPs, resulting in a slight increase in caspase-8 recruitment to the DISC (Unterkircher et al. 2011). Bortezomib increases DR5 protein levels in a p53-independent manner in glioma cells (Seol 2011). In this study, we sought to determine whether bortezomib at a dose capable of reducing cell viability and inducing apoptosis in T98G cells might have a specific effect on MGMT. Our results indicate that both protein and transcript levels of MGMT were attenuated after treatment and these effects coincided with a reduced total and nuclear amount of NFκB and with phosphorylation of the translation initiation factor 2alpha (eIF2α), thus revealing a role for bortezomib as an inhibitor of direct DNA repair both at the transcriptional and translational level in T98G glioblastoma cells.

Materials and Methods

Cell Culture and Reagents

The human glioblastoma cell line T98G was purchased from the European Collection of Animal Cell Cultures (ECACC, UK) and all experiments were performed within 6 months from purchase. The cell line was cultured in Dulbecco's modified Eagle's medium (GIBCO, UK) supplemented with 10 % heat-inactivated FBS (GIBCO, UK), 5 % L-glutamine (GIBCO, UK), and 1 % penicillin–streptomycin (Euroclone, UK) at 37 °C in a humidified 5 % CO2 atmosphere. Proteasome inhibitor (Bortezomib, VELCADE) was purchased from Janssen-Cilag Pharmaceuticals, Greece.

MTT Assay

Cells were plated in 96-well plates at a density of 2×10^5. Cells were allowed to attach for 24 h at 37 °C and were then either left untreated for another 24 h or were treated with bortezomib for 24 h at ten-fold serial dilution concentrations between 10^{-9} and 10^{-6} M. After incubations, 10 μL of MTT reagent (R&D Systems, UK) were added in each well, and plates were further incubated for 4 h at 37 °C. Then, 100 μL of MTT detection reagent (R&D Systems, UK) was added in each well, and plates were left at room temperature overnight, protected from light. Samples were analyzed at a wavelength of 570 nm with a reference at 650 nm in a 1420 Victor device (Wallac Victor, Finland). Experimental wells were carried out in triplicate ($p < 0.01$).

DNA Fragmentation

DNA fragmentation was determined using the cellular DNA fragmentation ELISA kit (Roche Diagnostics, Germany). After exposure to bromodeoxyuridine (BrdU) for 18 h, cells were reseeded onto a microplate (10^5 cells/well) and were then either left untreated for 24 h or were treated for 24 h with 0.1 μM bortezomib. Supernatant was removed and the remaining cells were lysed with the kit buffer. Cell lysate was transferred into an anti-DNA precoated microtiter plate and analyzed using the ELISA procedure. After 1 h of incubation, exonuclease treatment was carried out during 30 min. DNA fragmentation was measured spectrophotometrically (at 450 and 690 nm as the reference wavelengths) after the anti-BrdU-peroxidase conjugate and the substrate solutions had been added. BrdU-labeled fragments measured in cell lysate denoted apoptotic fragmentation. The results presented are the average value of three independent experiments ($p < 0.01$).

Western Blot Analysis

Fractionation of cells, analysis of nuclear and cytoplasmic fractions, or total cellular proteins, electrophoresis and immunoblotting were carried out as previously described (Patrikidou et al. 2011). Primary antibodies against the p65

subunit of NFκB and IκBα (Santa Cruz Biotechnology, USA), phospho-eIF2α (Ser51) and total eIF2α (Cell Signaling Technology, USA), Histone 2B (Abcam, UK), and actin (Sigma Aldrich, UK) were used. Treatment conditions were as described above. Densitometric analysis of the bands in blots was carried out with the public domain software for image analysis "ImageJ" (National Institute of Health, USA). All experiments were carried out in triplicate ($p < 0.01$) and representative results are shown.

20S Proteasome Activity Assay

Total protein cell lysates were prepared using a 0.5 % CHAPS buffer which did not affect proteasomal enzymatic activity. Chymotryptic activity of the 20S proteasome was measured in total cell lysates as previously described (Patrikidou et al. 2011). Treatment conditions were as described above. All measurements were performed in triplicate ($p < 0.01$).

RT–PCR

Total RNA was isolated using a commercial RNA isolation kit (Bio-Rad, USA). Using 1 mg total RNA, single-stranded DNA (cDNA) was synthesized by the use of a cDNA synthesis kit (Bio-Rad, USA). The primers used were, for MGMT forward, AGAGTCGTTCACCAGACAGG and MGMT reverse, GCCATTCCTTCACGGCCAG and for GAPDH forward, GGA AGG TGA AGG TCG GAG TCA and GAPDH reverse, GTC ATT GAT GGC AAC AAT ATC CAC, respectively. Conditions for MGMT were 35 cycles, Tanneal 55 °C and for GAPDH were 35 cycles, Tanneal 58 °C. PCR products were subjected to electrophoretic analysis on 2 % agarose gels containing ethidium bromide (Sigma Aldrich, UK), and were visualized and photographed under UV light. Treatment conditions were as described above. All experiments were carried out in triplicate ($p < 0.01$) and representative results are shown.

Statistical Analysis

The Graph Pad Instat Statistical package for Windows (GraphPad Software, USA) was used. Data are expressed as mean ± standard deviation (SD). The one-way analysis of variance with the Bonferroni post-test was used for the comparison of data, and the statistical significance limit was set at $p < 0.05$.

Results and Discussion

Proteasome inhibition by bortezomib has been previously shown to be active in MGMT-expressing glioblastoma cells (Yin et al. 2005; Tianhu et al. 2010; Unterkircher et al. 2011). In addition, some evidence of clinical activity was noted in phase I studies in patients with malignant gliomas (Kubicek et al. 2009; Phuphanich et al. 2010). The aim of this study was to investigate for a specific effect of the drug on MGMT expression in T98G cells, given that MGMT is the one of the major causes of glioblastoma resistance to treatment (Haar et al. 2012).

First, we performed an MTT assay and observed that bortezomib is capable of reducing cell viability as early as 24 h with the 50 % inhibiting concentration (IC50) of the

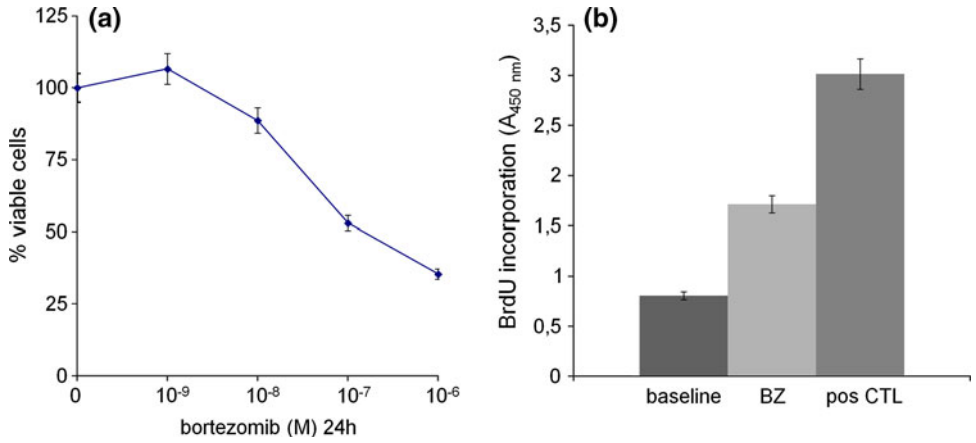

Fig. 1 Effect of bortezomib on cell viability and apoptosis of T98G cells. **a** MTT assay of T98G cells treated with dose-series incubations with bortezomib (BZ) for 24 h. Results are expressed as mean percentage of cell counts in control wells. Each point represents a mean ± SD of three independent experiments carried out in triplicate ($p < 0.01$). **b** DNA fragmentation by ELISA assay, as measured by absorbance (OD 450 values) at 24 h. Lanes from left to right: baseline untreated cells (incubated for 24 h with culture medium), bortezomib 0.1 μM for 24 h, positive apoptosis control. Each point represents amean ± SD of three independent experiments carried out in triplicate ($p < 0.01$)

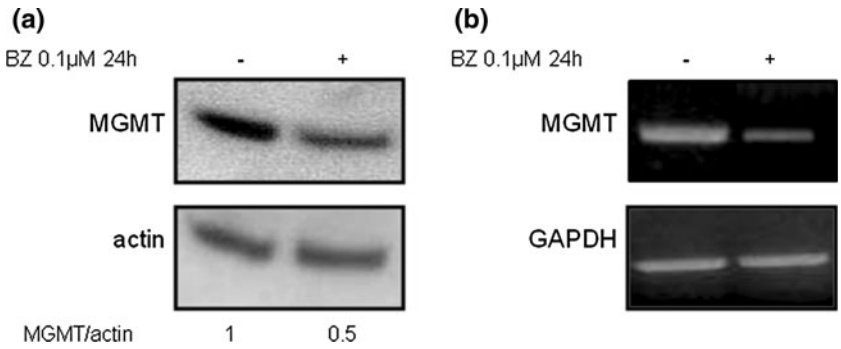

Fig. 2 Effect of bortezomib on MGMT expression. **a** Western blot analysis of MGMT protein levels at baseline, in untreated cells (incubated for 24 h with culture medium) (*left lane*) and after treatment with bortezomib (BZ) 0.1 μM for 24 h (*right lane*). **b** RT-PCR of MGMT mRNA levels at baseline (*left lane*) and after treatment with BZ 0.1 μM for 24 h (*right lane*)

drug at 24 h being approximately 0.1 μM (Fig. 1a). At this dose and time of administration, bortezomib was also found to induce DNA fragmentation and subsequent apoptosis in BrdU-based DNA fragmentation ELISA (Fig. 1b). We then investigated whether these effects might be, at least partially, related with abrogation of MGMT. Indeed, both protein (Fig. 2a) and transcript (Fig. 2b) levels of MGMT were found to be markedly reduced compared to untreated cells (Fig. 2). This was quite an unexpected and previously unreported finding, given that bortezomib, as a proteasome inhibitor, would be supposed to stabilize protein levels of proteasome substrates, including MGMT.

We hypothesized that this dual inhibition of MGMT mRNA and protein levels might involve an effect on transcription factors responsible for induction of MGMT gene with or without a secondary effect on the translational machinery. With regard to the former, our attention was drawn toward studying NFκB which not only is a key transcriptional inducer of MGMT gene expression (Lavon et al. 2007) but also has an established relationship with the ubiquitin–proteasome system and is constitutively activated in glioblastoma, promoting cancer cell migration, invasion and resistance to chemotherapy (Raychaudhuri et al. 2007). We show that bortezomib attenuates NFκB

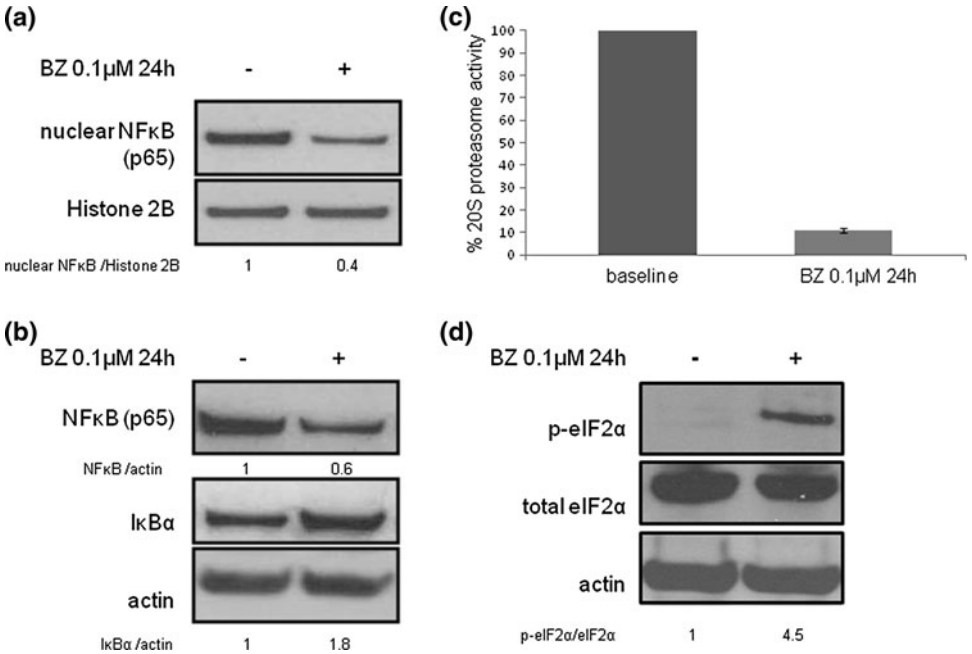

Fig. 3 Effect of bortezomib on the canonical NFκB pathway and eIF2α levels. **a** Western blot analysis of nuclear NFκB levels at baseline, in untreated cells (incubated for 24 h with culture medium) (*left lane*) and after treatment with bortezomib (BZ) 0.1 μM for 24 h (*right lane*). **b** Western blot analysis of total IκBα levels at baseline (*left lane*) and after treatment with bortezomib (BZ) 0.1 μM for 24 h (right lane). **c** Percentage of 20S proteasomal activity (calculated in RFU/mg) after treatment with bortezomib (BZ) 0.1 μM for 24 h (*right lane*) compared to baseline level (*left lane*). Each point represents a mean ± SD of three independent experiments carried out in triplicate ($p < 0.01$). **d** Western blot analysis of phosphorylated and total eIF2α levels at baseline (*left lane*) and after treatment with bortezomib (BZ) 0.1 μM for 24 h (*right lane*). The numbers located below each lane correspond to the quantification of the phosphorylated eIF2α signals by densitometry adjusted to the total eIF2α protein levels. The phosphorylation of eIF2α in the untreated group was arbitrarily set at the value of 1

nuclear accumulation (Fig. 3a) through increasing cellular IκBα protein levels (Fig. 3b) after inducing almost complete inhibition of 20S proteasome activity (Fig. 3c) in T98G cells. In addition, we have further observed a downregulation of total NFκB protein levels in response to bortezomib treatment (Fig. 3b). With regard to the decrease in MGMT protein levels, this might be another example of the pleiotropic effects of proteasome inhibition in cells. As such, proteasome inhibitors, including bortezomib, have been shown to cause a rapid decrease in the total protein synthesis rate due to phosphorylation of the alfa subunit of the eukaryotic translation initiation factor 2 (eIF2alpha) in several cancer cell types (Zhu et al. 2009; Yerlikaya and DoKudur 2010). We replicated and extended these findings in T98G cells, demonstrating a significant 4.5 fold-increase in the ratio of phosphorylated to total eIF2α levels after bortezomib treatment, compared to untreated cells (Fig. 3d).

To date, several other transcription factors and coactivators have been suggested to be involved in transcriptional regulation of MGMT, including p53, AP-1, Sp1, HIF1α, glucocorticoids, cAMP response element-binding protein, p 300 and MGMT enhancer binding protein (Bhakat and Mitra 2000; Biswas et al. 1999; Bocangel et al. 2009; Boldogh et al. 1998; Chen et al. 1997; Harris et al. 1996; Persano et al. 2012; Pieper 1997; Sato et al. 2011). In addition, post-transcriptional regulation of MGMT has also been reported through STAT3 signaling (Kohsaka et al. 2012) as well as through mRNA processing by microRNAs (miR), in particular miR-181d (Zhang et al. 2012). Their potential contribution in determining the final response of T98G cells to bortezomib treatment in terms of MGMT regulation is underscored here. Another limitation of our study is the use of only one cell line, which was however chosen on the basis of its high level of MGMT expression at baseline, compared to other human-derived glioblastoma cell lines (Hermisson et al. 2006). Finally, our data show an association between the decrease in the amount of nuclear and total NFκB and the decrease in the MGMT mRNA expression. Similarly, phosphorylation of eIF2α was also associated with reduced protein synthesis of MGMT. However, further work may be needed to fundamentally demonstrate the direct role of these phenomena in the decreased expression of MGMT.

In conclusion, our data suggest that bortezomib is an inhibitor of direct DNA repair, exerting a dual effect on MGMT, via attenuation of NFκB-inducible MGMT transcription and through eIF2α phosphorylation-dependent MGMT translational repression, respectively. This novel mechanism of bortezomib action regarding MGMT remains to be further elucidated and verified in studies with combination treatments including temozolomide or/and radiotherapy or/and antiangiogenic therapy, with the aim of reducing chemoresistance of glioblastoma and achieving better responses and outcomes for such patients.

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