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# Proteasome inhibitors sensitize glioma cells and glioma stem cells to TRAIL-induced apoptosis by PKCε-dependent downregulation of AKT and XIAP expressions ☆

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ABSTRACT

In this study we examined the effects of proteasome inhibitors on cell apoptosis in TRAIL-resistant glioma cells and glioma stem cells (GSCs). Treatment with proteasome inhibitors and TRAIL induced apoptosis in all the resistant glioma cells and GSCs, but not in astrocytes and neural progenitor cells. Since PKCε has been implicated in the resistance of glioma cells to TRAIL, we examined its role in TRAIL and proteasome inhibitor-induced apoptosis. We found that TRAIL did not induce significant changes in the expression of PKCε, whereas a partial decrease in PKCε expression was obtained by proteasome inhibitors. A combined treatment of TRAIL and proteasome inhibitors induced accumulation of the catalytic fragment of PKCε and significantly and selectively decreased its protein and mRNA levels in the cancer but not in normal cells. Overexpression of PKCε partially inhibited the apoptotic effect of the proteasome inhibitors and TRAIL, and the caspase-resistant PKCεD383A mutant exerted a stronger inhibitory effect. Silencing of PKCε induced cell apoptosis in both glioma cells and GSCs, further supporting its role in cell survival. TRAIL and the proteasome inhibitors decreased the expression of AKT and XIAP in a PKCε-dependent manner and overexpression of these proteins abolished the apoptotic effect of this treatment. Moreover, silencing of XIAP sensitized glioma cells to TRAIL. Our results indicate that proteasome inhibitors sensitize glioma cells and GSCs to TRAIL by decreasing the expression of PKCε, AKT and XIAP. Combining proteasome inhibitors with TRAIL may be useful therapeutically in the treatment of gliomas and the eradication of GSCs.



## 1. Introduction

Gliomas are the main primary central nervous system (CNS) tumors in humans, accounting for almost 80% of brain malignancies [1]. Despite advances in current treatment options including surgery, radiation therapy and chemotherapy, the prognosis for patients with these tumors continues to be poor with a median survival of 12–14 months [2]. The resistance of gliomas to chemotherapeutic drugs is one of the primary limitations to therapy [3], and therefore the identification of novel therapeutic approaches is urgently needed.

Recently, a small subpopulation of CD133+ cancer stem cells was identified in specimens of glioblastoma (GBM) [4,5]. These glioma stem cells (GSCs) express additional stem cell markers, exhibit self-renewal, the ability to differentiate to glial and neuronal lineages, and can initiate xenograft tumors which closely recapitulate the parental tumors [5]. GSCs have been implicated in enhanced chemo- and radio-resistance and in the repopulation of GBM tumors following these treatments [6,7]. In addition, a recent study demonstrated that GSCs are also resistant to TRAIL [8], thus, delineating the molecular mechanisms underlying the increased resistance of these cells to anti-cancer therapies is of utmost importance.

TRAIL, a type-II transmembrane protein, belongs to the tumor necrosis factor (TNF) superfamily [9]. Following binding of TRAIL to the TRAIL-R1 or -R2, the trimerized receptors recruit several cytosolic proteins that form the death-inducing signaling complex (DISC). This leads to the activation of caspase 8 at the DISC followed by either activation of a mitochondrial-independent pathway via caspases 3 and 7 or activation of a mitochondrial-dependent pathway by cleavage of Bid and activation of caspase 9 [10]. Importantly, TRAIL promotes apoptotic cell death in a variety of tumor cells but not in most normal cells, and is therefore considered a potentially attractive anticancer agent [11]. However, considerable numbers of cancer cells, including some glioma cells and GSCs, are resistant to the apoptotic effect of TRAIL [8,12]. Thus, understanding mechanisms that will abrogate the resistance of these cancer cells to TRAIL is important for the successful employment of TRAIL in cancer therapy.

Recent studies by us and others have shown that PKCε plays a role in the resistance of cancer cells to the apoptotic effect of TRAIL and in the survival of glioma cells [13–17]. PKCε is a member of the novel

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subgroup of the protein kinase C (PKC) family and plays important roles in the proliferation, differentiation and apoptosis of diverse cellular systems [18,19]. In addition to its activation by DAG, PKCε is activated by cleavage in the hinge region, which results in a constitutively active catalytic domain [20]. PKCε has been implicated in the regulation of both cell survival and apoptosis in various cellular systems [21,22].

Proteasome inhibitors have been reported to sensitize a variety of cancer cells to TRAIL-induced apoptosis [23–25]; however, the effect of these inhibitors in the sensitization of GSCs has not yet been reported. The molecular mechanisms underlying this sensitization are not completely understood and the sensitizing effects of the proteasome inhibitors bortezomib (PS-341) and MG-132 have been attributed to increased expression of the TRAIL death receptor, DR5 [26,27], a decrease in c-FLIP [23] and XIAP expression [28,29], activation of caspases or inhibition of NF-κB [24,30]. The role of PKCε in this sensitization has not yet been described.

In this study we examined the effects of proteasome inhibitors on the sensitization of glioma cells and GSCs to TRAIL focusing on the role of PKCε. We found that combined treatment with the proteasome inhibitors MG-132 or bortezomib and TRAIL induced apoptosis in TRAIL-resistant glioma cells and GSCs, without inducing toxicity to normal astrocytes and neural stem cells. In addition, the proteasome inhibitors and TRAIL induced both cleavage and decrease of PKCε mRNA and protein levels. The catalytic domain of PKCε exerts an apoptotic effect and the decrease in PKCε expression induces cell apoptosis by downregulating the expression of AKT and XIAP.

## 2. Materials and methods

### 2.1. Materials

Anti-PKCε antibody was purchased from Santa Cruz (Santa Cruz, CA). TRAIL was obtained from Peprotech (Rocky Hill, NJ), MG-132 was obtained from EMD (Gibbstown, NJ) and anti-active caspase 3, AKT and XIAP antibodies were purchased from Cell Signaling Technology (Beverly, MA). Bortezomib was generously provided by Millennium Pharmaceuticals, Inc. (Cambridge, MA) and the National Cancer Institute (Bethesda, MD).

### 2.2. Cell lines

The glioma cell lines U87-MG, LN229, LN-19, LNZ-308, A172 and U251 were obtained from ATCC (Manassas, VA). Normal astrocytes were obtained from Cambrex (Walkersville, MD) and grown in an astrocyte-specific medium provided by the manufacturer.

### 2.3. Primary glioma cultures

Primary cultures were obtained from freshly resected tissues 1 h after surgical removal as previously described [15]. Institutional Review Board-approved informed consent was obtained from all patients or from the patient's guardian for use of tumor tissue collected at the time of tumor resection.

### 2.4. GSCs and enrichment of CD133+ cells

The generation of GSCs, the enrichment of CD133+ cells and their characterization was recently described [31]. Spheroids were maintained in neurosphere medium and examined for the expression of CD44, Bmi-1, CD133, Musashi-1, Sox2 and nestin.

### 2.5. Neural progenitor cells

Neural progenitor cells (NPCs) were prepared and maintained as previously described [32]. Briefly, brains from 2-day-old rat pups were dissected and the striatum (with the adjacent subventricular zone [SVZ]) was removed. Following dissociation, the cells were plated in serum-free chemically defined medium [32] and basic fibroblast growth factor (10 ng/ml) was added daily to the neurospheres.

### 2.6. hESC-derived neural precursor cells

Human neural precursor cells containing neurospheres were generated as previously described from the human embryonic stem cell (hESC) line HUES 9 [33] by co-culturing the hESCs on a PA6 monolayer. PA6-hESC neurospheres expressed markers of neural precursors such as Sox1 and NCAM and of early neurons (β-III-tubulin, NCAM). Upon plating on laminin, immunocytochemical staining revealed that most neurospheres differentiated into neurons and GFAP+ cells within one week of culture.

### 2.7. Neurosphere formation assay

The ability of CD133+ cells to form secondary neurospheres was determined as previously described [31]. Briefly, disaggregated cells were treated with the appropriate treatments and cells were plated in 24-well plates at a density of 100 cells/well through limiting dilution. The number of neurospheres/well was determined 14 days thereafter for eight different wells. Spheres that contained more than 20 cells were scored. Results are presented as % of maximal neurospheres formed in control untreated cells.

### 2.8. Cell transfection

Cells were transfected by electroporation using the Nucleofector device, protocol number U29 (Amaxa Biosystems, Gaithersburg, MD). Transfection efficiency using nucleofection was about 70% to 90%.

For the silencing experiments cells were transfected with SureSilencing XIAP, PKCε or control shRNA plasmids (SuperArray, Frederick, MD). Prior to transfection, the GSC spheroids were mechanically dissociated to smaller spheroids, and transfection was performed by electroporation using the Nucleofector device program A027 and the mouse NSC Nucleofector kit (Amaxa Biosystems, Gaithersburg, MD), as previously described [31]. In some experiments we employed siRNA duplexes targeting PKCε or XIAP and a control scrambled sequence (Thermo Scientific, Lafayette, CO). Transfection of siRNAs (50 nM) was performed using OligofectAMINE (Invitrogen, Carlsbad, CA) as previously described [15].

### 2.9. Site-directed mutagenesis of PKCε

PKCε and the caspase-resistant mutant PKCεD383A were prepared and characterized as previously described [15]. pcDNA-AKT1-HA and pcDNA-XIAP-Myc were obtained from Addgene (Cambridge, MA).

### 2.10. Measurements of cell apoptosis

Cell apoptosis was measured using propidium iodide staining and analysis by flow cytometry as previously described [34] and by Western blot analysis of active caspase 3 using anti-cleaved caspase 3.

Cell death was also quantitatively assessed by measuring lactate dehydrogenase (LDH) levels in the medium using the LDH kit according to the manufacturer's protocol.

### 2.11. Intracellular staining of active caspase 3 using flow cytometry

Cells were fixed with 2% formaldehyde for 10 min and permeabilized on ice with 90% methanol for 30 min. The cells were then incubated with anti-cleaved caspase-3 antibody for 1 h at room temperature. Following washes, the cells were incubated with FITC-conjugated anti-rabbit immunoglobulin for an additional 30 min. Stained cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer with CellQuest software.