Marizomib (NPI-0052) Activity as a Single Agent in Malignant Glioma

Kaijun Di[1], Xing Gong[2], Dana M. Curticiu[3], Michael Palladino [4], and *Daniela A. Bota[1,2,5]

(1) Department of Neurological Surgery, UC Irvine School of Medicine, (2) Department of Neurology, UC Irvine School of Medicine; (3) Department of Plant Breeding and Genetics, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania; (4) Triphase Accelerator U.S. Corporation, San Diego, CA; (5) Chao Family Comprehensive Cancer Center, UC Irvine, USA

INTRODUCTION

Glioblastoma multiforme (GBM) is a very aggressive tumor and highly resistant to conventional chemotherapy. The current chemotherapeutic agents aim at inducing extensive DNA damage and disrupting the mitotic machinery of the cells, and affect not only the malignant cells but also the normal neural tissues, generating long-standing neurotoxicity in cancer survivors.[1]

The ubiquitin proteasome pathway is a key regulator in maintaining cellular homeostasis. It is responsible for the degradation of intracellular proteins including not only denatured, misfolded, and or aged proteins, but those that regulate critical signaling pathways. Defects within these pathways are associated with a number of diseases, including cancer. Pre-clinical studies have demonstrated that malignant cells are more susceptible to the cytotoxic effects of proteasome inhibition than normal cells, leading to the development of a powerful anti-cancer strategy by targeting proteasome. Bortezomib (PS-341, Velcade®) is the first proteasome inhibitor that had been approved by FDA for the treatment of multiple myeloma and subsequently mantle cell lymphoma. A second irreversible proteasome inhibitor carfilzomib which has a limited 20S proteasome inhibition profile was recently approved by FDA for the treatment of multiple myeloma. Although effective in the treatment of extracranial cancers, carfilzomib and bortezomib have a limited capability to cross the blood brain barrier (BBB) at efficacious doses and therefore may have only modest activity as a treatment of malignant gliomas.

Marizomib (NPI-0052, Salinosporamide A) is a second generation irreversible proteasome inhibitor whose bicyclic β-lactone g-lactam structure differs significantly from other peptide based proteasome inhibitors such as bortezomib and carfilzomib. Marizomib has a broader inhibition profile for the 20S proteasome, and has been shown to inhibit the chymotrypsin-like (CT-L, β5), caspase-like (C-L, β1) and trypsin-like (T-L, β2) activities of the 20S proteasome. Marizomib has also been found to activate a variety of the caspases i.e., 3, 8 and 9, secondary to the buildup of ROS (Reactive oxygen species) and misfolded proteins in the cells after inhibition of proteasome activity, and thus to induce apoptotic cell death.[2]

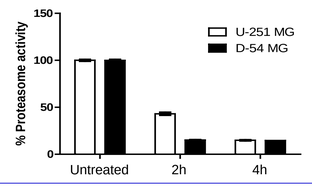
The purpose of this study was to determine the effect of marizomib on different types of serum-free stem cell cultures derived from human primary brain tumors, normal neural stem cells as well as stable malignant glioma cell lines, and to define the mechanism by which it operates in these models.

METHODS

- **Drug:** Marizomib (NPI-0052) was provided by Michael Palladino (Triphase Accelerator U.S. Corporation).
- **Cell lines:** The primary brain tumor stem-like cells (GSCs) (low-grade glioma DB29, DB30; high grade glioma DB17, DB26, DB32, DB33), Meningioma-SC-M1, Meningioma-SC-M2 and neural stem progenitor cells (NSCs) SC27 and DB31 were isolated from patients as previously described.[3] All the protocols were approved by the Institutional Review Board at University of California Irvine and Children's Hospital of Orange County. The established malignant human glioma cell lines, U-251 MG and D-54 MG, were gifts from Dr. Darrel Bigner at Duke University. The culture condition was same as previously described.[3]
- **20S proteasome activity assay**: The proteasomal chymotrypsin-like (CT-L) activity was measured using the Proteasome Activity Assay kit as recommended by the manufacturer (Chemicon International Inc.).
- **Wound closure assay:** Cells were grew to full confluency. Similar sized wounds were then induced to monolayer cells by scraping a gap using a micropipette tip. The time required for 'wound closure' was monitored and photographed immediately after wound incision and at indicated time points.
- **Invasion assay:** Invasion assay was performed using BD BioCoat™ Matrigel™ Invasion Chamber with an 8μm PET membrane (BD Biosciences).
- **Apoptosis assay:** The percentage of cells that were live, apoptotic, and dead was measured using the FITC Annexin V Apoptosis Assay Kit (BD Biosciences). Fluorescent (FITC) labeled Annexin V was used to tag cells that had undergone externalization of phosphatidylserine. Samples were then analyzed by flow cytometry using B.D. Bioflow. Results are expressed as the percentage of untreated cells.
- **Caspase assay:** The induction of Caspase-3 in marizomib treated D-54 MG cells was measured using the Apopcyto Caspase-3 Fluorometric Assay Kit (MBL International Corp.).
- **ROS measurements:** D-54 MG cells were treated with marizomib at 60nM for 18 hours in the presence or absence of 10mM NAC (N-acetyl cysteine). The intracellular ROS were measured by using the OxiSelect ROS Assay kit (Cell Biolabs, Inc.). The data was reported as RFU and normalized to 10,000 cells.
- **Antibodies for Western blotting:** Antibodies used were Cleaved Caspase-3 (Asp175) (#9661, Cell Signaling), cleaved p85 PARP (Y34, NB110-57321, Novus, CO, USA), and β-actin (NB600-501, Novus).
- **Statistical analysis:** Statistical analyses were performed and graphs generated using Prism 4.0 (GraphPad). All values were presented as mean standard error of the mean (S.E.M.). Statistical significance was measured by simple paired *t*-tests or one-way ANOVA.

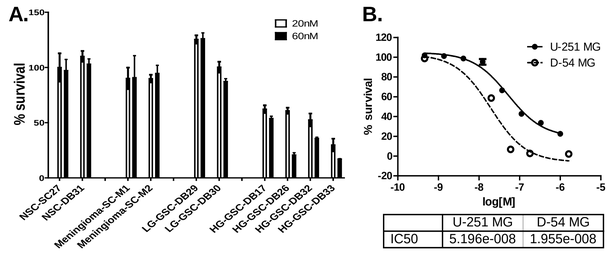
RESULTS

Figure 1. Marizomib inhibits proteasome activity in glioma cells.



Proteasome activity was measured in cell extracts at baseline, 2 hours and 4 hours after treatment with marizomib (60 nM).

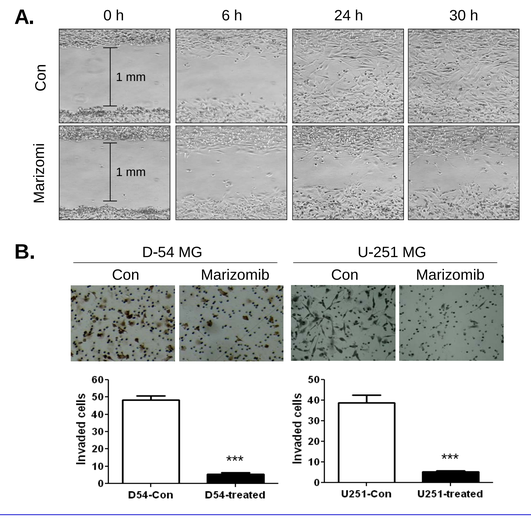
Figure 2. Malignant GSCs and established glioma cell lines are more sensitive to marizomib-induced proteasome inhibition than NSCs, low-grade GSCs and primary meningioma cultures.



	U-251 MG	D-54 MG
IC50	5.196e-008	1.955e-008

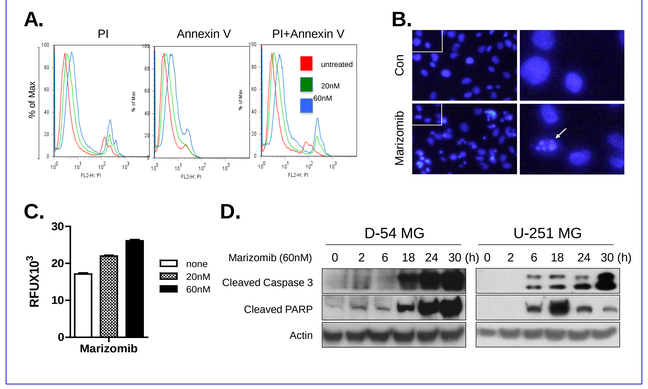
(**A**) Treatment with 20nM and 60nM of marizomib for 72 hours killed 50%-90% of the high-grade (DB17, DB26, DB32, DB33) GSCs. The same dose had limited toxicity for NSCs (SC27 and DB31), low-grade GSCs (DB29, DB30), and meningioma primary cultures. (**B**) Marizomib effectively reduces survival of malignant glioma established cell lines D54-MG and U251-MG.

Figure 3. Marizomib inhibits the motility of glioma cells.



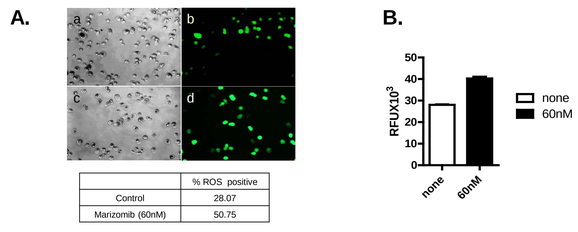
(**A**) Wound closure assay was performed using U-251 MG cells with or without 60nM marizomib treatment. The time required for 'wound closure' was monitored and photographed at indicated time points. (**B**) Invasion capability of the cells treated or untreated with 60nM marizomib for 24 hours was analyzed using Matrigel Invasion chambers. The average of invaded cells for each counting grid was showed in lower panel. ***$p < 0.001$.

Figure 4. Marizomib induces apoptosis and Caspase-3 activation in glioma cells.



(**A**) FITC Annexin V Apoptosis Assay kit was used to detect apoptosis in D-54 MG cells induced by marizomib (60nM) treatment for 24 hours. (**B**) After treated with marizomib (60nM) for 24 hours, DAPI staining was performed to observe apoptotic U-251 MG cells indicated by small, condensed nuclei. (**C**) Caspase-3 activity was measured in cell extracts, 24 hours after treatment with marizomib (60nM). An 80% increase in caspase-3 activity was found in treated group. (**D**) D54-MG and U251-MG were treated with 60nM marizomib for indicated timepoints. Cells were then collected and Western blot was used to detect cleaved Caspase-3 and PARP. Actin was the internal control.

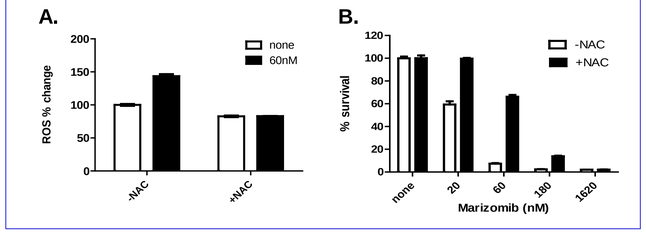
Figure 5. Marizomib increases ROS generation in glioma cells.



	% ROS positive
Control	28.07
Marizomib (60nM)	50.75

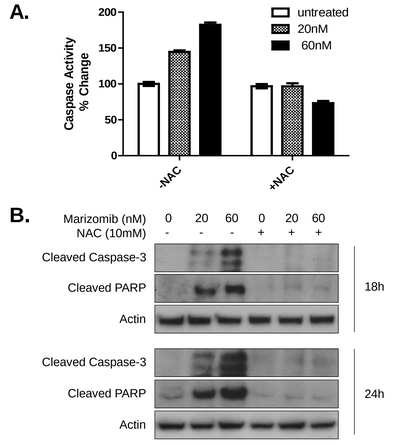
(**A**) ROS generation was measured as the number of DCF fluorescent cells by direct fluorescent microscopy. The percentage of ROS positive cells almost doubled after marizomib treatment. (**B**) Carboxy-H2DCFDA detectable ROS were measured by fluorescence spectroscopy. Values represent the mean ± SD of three experiments.

Figure 6. NAC pretreatment quenches ROS induction, and increases glioma cell survival upon marizomib treatment.



(**A**) The addition of the antioxidant NAC (10mM) blocked the marizomib-induced ROS activation in D-54 MG cells. (**B**) NAC pretreatment was able to rescue the D-54 cells from marizomib-induced cell death.

Figure 7. NAC blocks Caspase-3 activation induced by marizomib.



(**A**) The Caspase-3 activation caused by 60nM marizomib treatment was abolished by NAC addition. (**B**) D-54 MG cells were treated with 20nM or 60nM marizomib with or without 10mM NAC for indicated timepoints. Cells were then collected and Western blot was used to detect cleaved Caspase-3 and PARP. Actin was the internal control.

CONCLUSION

- Marizomib is an effective *in-vitro* agent, able to inhibit cell proliferation and motility in malignant glioma cells.
- Marizomib activates Caspase-3 and causes apoptotic cell death.
- A major mechanism of action for marizomib-induced apoptosis in glioma cells is ROS generation, which could be blocked by NAC pretreatment.
- Marizomib selectively kills high-grade GSCs and that the NSCs are relatively resistant to this drug, indicating that marizomib might be to offer malignant glioma tumor control with limited neurotoxicity.

REFERENCE

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2. Miller, C. P., Ban, K., Dujka, M. E., McConkey, D. J., Munsell, M., Palladino, M., and Chandra, J. (2007). NPI-0052, a novel proteasome inhibitor, induces caspase-8 and ROS-dependent apoptosis alone and in combination with HDAC inhibitors in leukemia cells. *Blood* 110, 267-277.
3. Kaijun Di, Mark E. Linskey and Daniela A. Bota. (2013) TRIM11 is over-expressed in high-grade gliomas and promotes proliferation, invasion, migration and glial tumor growth. *Oncogene* 32, 5038-5047.