

EZH2 as a Therapeutic Target in Glioblastoma: A Cellular and Molecular Study

Javier de la Rosa¹, Marta Iraburu¹, Gabriel Gallo-Oller¹, Mehdi H Shahi², Bárbara Meléndez³, Juan A Rey⁴, Miguel A Idoate⁵, and Javier S Castresana^{1*}

¹Department of Biochemistry and Genetics, University of Navarra School of Sciences, Pamplona, Spain

²Interdisciplinary Brain Research Centre, Faculty of Medicine, Aligarh Muslim University, Aligarh, India

³Molecular Pathology Research Unit, Virgen de la Salud Hospital, Toledo, Spain

⁴IdiPaz Research Unit, La Paz University Hospital, Madrid, Spain

⁵Department of Pathology, University of Navarra Clinic, Pamplona, Spain

*Corresponding author: Javier S Castresana, Department of Biochemistry and Genetics, University of Navarra School of Sciences, U Irunlarrea, Pamplona, 31008, Spain, Tel: +34-948-425600\806486; E-mail: jscastresana@unav.es

Received date: November 07, 2016; Accepted date: November 30, 2016; Published date: December 02, 2016

Copyright: © 2016 De la Rosa J, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Glioblastoma is the most common malignant brain tumor in adults and it is currently treated with a combination of surgery, radiotherapy and chemotherapy with temozolomide (TMZ). Many patients show resistance to TMZ, which is a challenge in the treatment of this type of brain cancer. New strategies are being tested, like the inhibition of EZH2, a histone methyltransferase which is overexpressed in cancer cells, leading to angiogenesis and metastasis. In this work, the EZH2 inhibitor DZNep was tested in A172 glioblastoma cells and in A172-TMZ-resistant glioblastoma cells. Inhibition of cell proliferation, adhesion, colony formation, and migration was noted in control and TMZ-resistant glioblastoma cells after DZNep treatment. At the level of EZH2 target gene expression, DZNep decreased EZH2 expression, and increased the expression of its target genes (E-cadherin and TIMP3), which might probably contribute to inhibiting the development of a cancer metastatic phenotype. Finally, DZNep negatively regulated the TGF β pathway. In conclusion, we propose that inhibition of EZH2 might be considered as a therapeutic strategy against glioblastoma.

Keywords: EZH2; DZNep; Glioblastoma; Brain tumors; Temozolomide; TGF β

EZH2 and DZNep

EZH2 is the catalytically active component of PRC2 (polycomb repressive complex 2), capable of trimethylating lysine 27 of histone H3 (H3K27met3) when in complex with SUZ12 and EED [7]. EZH2 is overexpressed in various cancers such as ovarian carcinoma [8] and prostate cancer [9] and it predicts poor prognosis, high tumor grade and high clinical stage [10]. Pre-clinical studies showed that EZH2 is able to silence several anti-metastatic genes (E-cadherin and tissue inhibitors of metalloproteinases), thereby favoring cell invasion and anchorage-independent growth [10]. In addition, EZH2 seems to play a crucial role in favoring tumor angiogenesis.

EZH2 is able to inhibit the expression of DAB2IP, which codes for a Ras-GTPase-activating protein. This event leads to increased metastatic potential through Ras- and NF- κ B-dependent pathway activation. Interestingly, the Ras-Mek-Erk pathway triggers EZH2 phosphorylation and activation, suggesting a positive feedback loop in cancer cells. EZH2 is at the crossroad of a complex microRNA network [11]. Several studies showed that miR-101, let7, and miR-26 inhibit EZH2 expression in cancer cells [12]. miR-101 is decreased in glioblastoma, thereby increasing EZH2 expression [13].

As a result of EZH2 activation, several genes are silenced: Vasohibin1 (VASH1) (a soluble inhibitor of tumor angiogenesis), E-cadherin (involved in adhesion), and TIMP3 (metalloproteinase inhibitor). This leads to increased metastasis and angiogenesis [10]. Furthermore, EZH2 is physically and functionally linked to DNA methylation and histone deacetylation, suggesting a collaboration among different epigenetic silencing enzymes [11,14,15].

Introduction

Glioblastoma [1,2] is the most common form of malignant brain cancer in adults, and it represents 12-15% of all intracranial tumors, and 50-60% of astrocytic tumors. Although very uncommon, it is highly lethal with the worst prognosis of any brain tumor and a 5-year survival rate of only 5% [1]. The median survival is less than 15 months [3]. Currently, the treatment of glioblastoma includes surgery, radiation therapy and chemotherapy. Although addition of temozolomide (TMZ) to radiotherapy has resulted in an overall increase in survival of glioblastoma patients, therapy still fails in most cases, due to incomplete tumor resection, and/or to the apparent resistance of tumor cells to irradiation and TMZ. Some tumors are insensitive to TMZ already at diagnosis, while others develop acquired TMZ resistance during treatment. Therefore, TMZ resistance and radiotherapy resistance represent a major challenge in the treatment of this disease [3-5].

The molecular marker which predicts the therapeutic effect of TMZ is Methyl Guanyl methyltransferase (MGMT), a DNA repair enzyme [6] that removes the methyl group from the O⁶ position of guanine caused by TMZ. Lack of expression of MGMT due to MGMT promoter methylation associates to a good TMZ response to treatment, and patients with unmethylated MGMT promoter show resistance to TMZ [6].

EZH2-dependent methylation requires the S-adenosyl-L-homocysteine (SAH) cofactor. DZNeP (3-dezaneplanocin-A), a SAH-hydrolase inhibitor, is a drug with cancer cell-specific antiproliferative activity. DZNeP demonstrated antitumor activity against breast, lung, brain, prostate, and liver cancer cells. In addition, DZNeP proved to block cancer cell migration and invasion in prostate cancer cells. Moreover, DZNeP treatment reduced tumor-associated blood vessel formation in a glioblastoma xenograft model, suggesting an *in vivo* anti-angiogenic activity [10].

EZH2 and the TGF β 1 pathway

Rao et al. [8] found that EZH2 knockdown reduces transforming growth factor β 1 (TGF β 1) expression, and increases E-cadherin expression. Furthermore, they observed a positive correlation between overexpression of EZH2 and TGF β 1 in ovarian carcinoma tissues [8]. Previous studies have shown that this pathway is involved in glioblastoma aggressiveness [16].

The TGF β pathway [17] involves the ligands (TGF β 1, TGF β 2, TGF β 3), the receptors (TGF β R1, TGF β R2) and the SMAD transcription factors. The binding of the ligands to the receptors leads to the phosphorylation of several SMADs, which form heteromeric complex that translocate to the nucleus where they associate with DNA-binding cofactors and target different sets of genes. This pathway is highly regulated by SMAD6 and SMAD7, which negatively control signal transduction [18]. SMADs have been shown to be overexpressed in some tumors [19]. Phosphorylated SMAD2 levels have been proposed as a negative prognostic marker in glioblastoma [20]. Another regulator of this pathway is BAMBI, a transmembrane protein which binds TGF β ligands but does not transduce any signal to the intracellular environment, therefore negatively regulating TGF β signaling [19]. Finally, another TGF β regulator is the isoform of the TGF β receptor, TGF β R3, which releases the soluble extracellular domain, known as sTGF β R3, and may sequester ligands to inhibit TGF β signaling [21].

The objective of this study is to test the effectiveness of DZNeP as a treatment against glioblastoma (in a control glioblastoma cell line and in a TMZ-resistant glioblastoma cell line). The effect of DZNeP on cell proliferation, adhesion, colony formation, and cell migration, together with gene expression of EZH2 targets and of TGF β 1 regulators is tested.

Materials and Methods

Cell line culture

A172 glioblastoma cells were grown in RPMI/GlutaMAXTM medium supplemented with heat-inactivated 10% fetal bovine serum (GIBCO, Paisley, UK), 1% penicillin/streptomycin (GIBCO, Paisley, UK) and 0.1% amphotericin B (GIBCO, Paisley, UK). These cells were grown as a monolayer in 75 cm² flasks at a confluence of 80-90% at 37°C and 5% CO₂.

Temozolomide resistant cell line

A172 cells were cultured at a 50% confluence. TMZ was added to the culture medium at 535 μ m. This medium was removed after 72 h, and new medium was added. Most of the cells died, but after 2 weeks some cells formed colonies. They were allowed to grow, then trypsinized and seeded in a new flask, where they were exposed to a

second dose of TMZ at 535 μ m which was removed after 72 h, as in the first exposure. The cells formed colonies. These are the cells which were used as a TMZ-resistant cell line during this study. After 20 culture passages, it is recommended to give a reminder dose of TMZ at 100 μ m, although it was not needed for this study.

DZNeP treatment

A172 control cells and A172-TMZ-resistant cells were exposed to DZNeP at a concentration of 5 μ m for 72 h. Both cell lines were used in the following experiments to analyze the effect of the drug on both, control and TMZ-resistant A172 glioblastoma cells.

MTT Assay

In order to test the sensitivity to DZNeP of the control A172 cell line and the TMZ-resistant A172 cell line, cells were exposed to different concentrations of the drug at 24 h and 48 h. For this purpose cells were seeded at 1000 cells in 200 μ l medium/well in a 96-wells flat-bottom plate, and exposed to different concentrations of DZNeP (1 μ m, 2.5 μ m, 5 μ m, 10 μ m, 15 μ m, 30 μ m, 50 μ m, 100 μ m) dissolved in DMSO. A control of DMSO to see the effect of the dissolvent on the cells was included in the study. Two plates with the following scheme were prepared: one of the plates was incubated for 24 h and the other one for 48 h, at 37°C and 5% CO₂. After that time, 50 μ l at 2 mg/ml of MTT (thiazolyl blue tetrazolium bromide) were added to each well; so the final concentration was 0.5 mg/ml. The plate was incubated for 3 h at 37°C, 5% CO₂ in darkness. After incubation, the medium with MTT was removed and 150 μ l of DMSO were added to each well. The absorbance was monitored at 550 nm using Multiskan EX. The data obtained were represented in a graph and analyzed.

Cell adhesion assay

The goal of this assay was to test the adhesion capacity of A172 glioblastoma cells at 4 different conditions: control A172, control A172 treated with DZNeP, A172-TMZ-resistant, and A172-TMZ-resistant treated with DZNeP. For this purpose, 7000 cells in 200 μ l of medium were seeded in each well. The plate was divided in 4 sections of 6x4 wells. The medium was not changed in the first section, in the second section the medium was changed after 15 min, in the third section the medium was changed after 30 min, and in the fourth section the medium was changed after 60 min. After this first h, 50 μ l at 2 mg/ml of MTT (thiazolyl blue tetrazolium bromide) were added to each well, so the final concentration was 0.5 mg/ml. The plate was incubated for 3 h at 37°C, 5% CO₂ in darkness. After incubation, the medium with MTT was removed and 150 μ l of DMSO were added to each well. The absorbance was monitored at 540 nm using Multiskan EX. The data obtained were represented in a graph and analyzed.

Colony formation assay

In this assay, the colony formation capacity of A172 at different conditions is tested (control A172, control A172 treated with DZNeP, A172-TMZ-resistant, and A172-TMZ-resistant treated with DZNeP). For each condition, 3 Petri plates with 1000 cells in 10 ml of medium each were incubated for 10 days at 37°C and 5% CO₂ in darkness. After this time, the medium was removed; the plates were washed with PBS, and incubated with 4% paraformaldehyde for 30 min into an extraction hood. After incubation with paraformaldehyde, plates were washed with PBS and stained with crystal violet (dissolved in water and ethanol) for 15 min. Then, crystal violet was removed and the

plates were washed with water and air dried. Finally the colonies were counted.

Scratching assay or wound healing assay

In this assay for cell migration capacity, 700,000 cells were seeded per well in a 6 well plate, with one well for each of the 4 following conditions: control A172, control A172 treated with DZNeP, A172-TMZ-resistant, and A172-TMZ-resistant treated with DZNeP. They were allowed to achieve confluence in 24 h, and then a wound was made on the monolayer by scratching it with a pipette tip. Pictures were taken after 4, 8, 18, 30 and 42 h for cell migration testing.

RNA and protein extraction

For the isolation of RNA and proteins from the A172 glioblastoma at the 4 different conditions (control A172, control A172 treated with DZNeP, A172-TMZ-resistant, and A172-TMZ-resistant treated with DZNeP), the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) was used. Proteins were kept at -20°C, and RNA was kept at -80°C. Nanodrop was used to determine the purity and concentration of RNA.

Quantitative RT-PCR

For retrotranscription, 2 µg of RNA were mixed with 1 µl of random primers at 250 ng/µl and 2 µl of dNTPs mix at 5 µm in a final volume of 12 µl of water. This mixture was incubated for 5 min at 65°C. Then, 4 µl of synthesis buffer and 2 µl of DTT were added, and this was incubated for 2 min at 42°C. Then, 1 µl of SuperScript Reverse Transcriptase was added for the synthesis of cDNA and this final mixture was incubated 10 min at 25°C, 50 min at 42°C, and finally 15 min at 72°C. 80 µl of water were added and the cDNA was stored at -20°C.

Real time PCR was used to analyze gene expression. The analyzed genes were (Table 1): TIMP3, E-cadherin, EZH2, SMAD6, SMAD7, TGFβ1, TGFβ2, TGFβ3, BAMBI. Genes related to EZH2 (TIMP3, E-cadherin, EZH2) were analyzed in the 4 different cell conditions: control A172, control A172 treated with DZNeP, A172-TMZ-resistant, and A172-TMZ-resistant treated with DZNeP. Genes related to TGFβ1 (SMAD6, SMAD7, TGFβ1, TGFβ2, TGFβ3, TGFβ3, BAMBI) pathway were analyzed in control A172, and in control A172 treated with DZNeP.

GENE	SEquence 5'-3'	TM (°C)
HPRT-fw	TGACACTGGCAAAACAATGCA	60
HPRT-rv	GGTCCTTTTCACCAGCAAGCT	60
GAPDH-fw	AACGTGTCACTGGTGGACCTG	60
GAPDH-rv	AGTGGGTGTCGCTGTTGAAGT	60
B-actin-fw	TCTACAATGAGCTGCGTGTG	60
B-actin-rv	GGTGAGGATCTTCATGAGGT	60
B-tubulin-fw	AGCCAGCAGTGCTAAACCC	60
B-tubulin-rv	TGACAGCAATAGATTTATTAAGTATCCC	60
EZH2-fw	CAGTAAAAATGTGTCCTGCAAGAA	60
EZH2-rv	TCAAGGGATTCCATTCTCTTCGA	60

E-cadherin-fw	GGAGGAGAGCGGTGGTCAAA	60
E-cadherin-rv	TGTGCAGCTGGCTAAGTCAA	60
TIMP3-fw	TCTGCAACTCCGACATCAGT	60
TIMP3-rv	TTGGTGAAGCCTCGGTACAT	60
smad7-fw	CCAACTGCAGACTGTCCAGAT	60
smad7-rv	ATGCCACCACGCACCAGTGT	60
smad6-fw	AATCTCCGCCACCTCCCTAC	64
smad6-rv	GAATTCACCCGGAGCAGTGA	64
TGFBRc1-fw	CGTCAGGTTCTGGCTCAGGTT	60
TGFBRc1-rv	TCTGCCTCACGGAACCACGAA	60
TGFBRc2-fw	ACGTTCAGAAGTCGGATGTGG	64
TGFBRc2-rv	TGTGAAACTTGACTGCACCGT	64
TGFB2-fw	ATCGAAGGAGAGCCATTCGC	64
TGFB2-rv	GCCTGAACAACGGATTGAGC	64
TGFB3-fw	CTCCAGCCAGATCCTGTCT	64
TGFB3-rv	ATGATGATTCACCCACACCG	64
BAMBI-fw	AGCTACATCTTCATCTGGCTGC	64
BAMBI-rv	CATGGGTGAGTGGGGAATTTG	64

Table 1: Sequences and melting temperatures (TM) of the primers used for qRT-PCR.

The primers were analyzed with Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) in order to obtain product length, %GC, melting temperature (Tm) and check for the specificity of the template. In the beginning HPRT (Table 1) was used as a housekeeping gene, but its expression varied among the samples. Therefore the genes GAPDH, β-tubulin and β-actin (Table 1) were tested as housekeeping candidate genes, being β-actin the chosen gene.

The amplification step and melting curves were carried out in IQ5 Multicolor real-time PCR detection system (BIO-RAD). The reaction mix contained 12.5 µl of 2X IQ[™] SYBR[®] Green Supermix (BIO-RAD), 0.5 µl of forward primer, 0.5 µl of reverse primer, 1.5 µl cDNA and 10 µl of water. An initial denaturation step at 95°C for 10 min was followed by 40 cycles of amplification alternating between 95°C for 15 s, then 30 s at corresponding annealing temperature for each gene and 30 s at 72°C. After the amplification, a melting curve was added as a quality control and it followed the next steps: from 70°C to 90°C, 30 s at every 0.5°C. All samples were run in triplicate. The 2^{ΔΔCt} method was used to analyze the relative gene expression.

Western blot

Western blot was performed to assess protein expression of SMAD2 (phosphorylated and non-phosphorylated), BAX, GAPDH and as a housekeeping β-actin. Total proteins were extracted as previously described (AllPrep kit). The protein concentration of the samples was determined with the BCA protein assay kit. 30 µg of total protein were resolved by SDS-PAGE under reducing conditions and then transferred to a nitrocellulose membrane. This membrane was blocked

with TBS containing 0.1% Tween 20 and 5% non-fat milk for 1 h. TBS-tween 0.1% was used to wash the blots and then they were incubated with primary antibody. After this incubation, 3 washes were made and blots were incubated with the secondary antibody. In order to detect the secondary antibody, chemiluminescence and autoradiography were performed. Finally, the intensity of the bands was quantified by ImageJ. Antibodies used for Western blot are represented in Table 2.

Antibody	Kd	Speci e	Dilution	INCUBATIO N	Type
antiSmad2	60	Rabbit	1/4.000	O/N (4°C)	Primary
antiPhosphoSmad 2	60	Rabbit	1/2.000	O/N (4°C)	Primary
anti-Bax	20	Rabbit	1/2.000	O/N (4°C)	Primary
anti-GAPDH	37	Mous e	1/20.000	1h (RT)	Primary
anti-β-actin	42	Mous e	1/20.000	1h (RT)	Primary
ANTI-MOUSE iGg-hrp	-	Goat	1/40.000	1h (RT)	Secondar y
ANTI-RABBIT IGG-H&I	-	Goat	1/10.000,1/3.00 0	1h (RT)	Secondar y

Table 2: Primary and secondary antibodies used for Western blots. RT: room temperature. O/N: overnight.

Statistical analysis

Data were analyzed by parametric methods. When comparing more than 2 samples ANOVA test was used, followed by Dunnet as a post test. When comparing 2 samples a t test was performed. All these analyses were carried out with the program GraphPad.

Results

DZNeP decreases cell proliferation in control and TMZ-resistant glioblastoma cell lines

The MTT assay is a colorimetric assay designed for measuring cell proliferation. This experiment revealed that the TMZ-resistant cell line responds to DZNeP in a similar way to the control cell line. The statistical analysis showed that with a 24 h exposure only high doses (100 μm) achieved a significant decrease in the number of cells. However, with a 48 h exposure the lowest dose (1 μm) was enough to produce a significant decrease in proliferation (Figure 1A).

DZNeP decreases cell adhesion in control and TMZ-resistant glioblastoma cell lines

The MTT assay, in this case was performed in order to test the adhesion capacity of the different cells. This experiment showed a decrease on the capacity of adhesion of cells treated with DZNeP, compared to non-treated cells in both control and TMZ-resistant cells. This effect is seen most clearly at 60 min (Figure 1B).

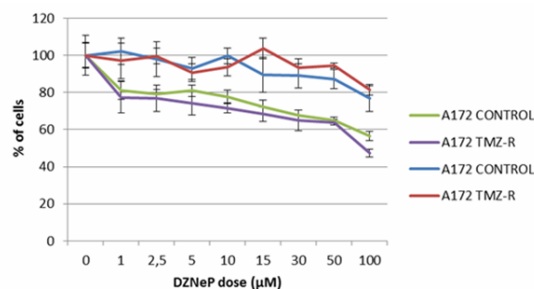


Figure 1A: MTT assay after 24 and 48 h exposure to DZNeP of glioblastoma cell lines (A172, control; and A172-TMZ-R, resistant to temozolomide). A 24 h exposure only caused a significant decrease in proliferation at the highest concentration, while the lowest dose was enough to provoke a significant decrease in proliferation after 48 h exposure.

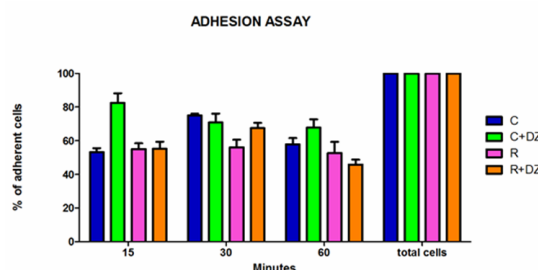


Figure 1B: Adhesion assay, showing a decrease in the adhesion capacity of both cells treated with DZNeP for 15 min to 60 min: A172 cells (control, C), and A172-TMZ-R cells (temozolomide resistant, R).

DZNeP decreases colony formation in control and TMZ-resistant glioblastoma cell lines

The aim of a colony formation assay is to test and compare the capacity of the cells to form colonies, as this relates to *in vitro* tumorigenicity. The number of colonies formed by cells treated with DZNeP was significantly lower than the number of colonies formed by non-treated cells, especially in the TMZ-resistant cell line (Figure 1C). It is also visible that TMZ-resistant cells have a higher colony formation capacity than the control cells.

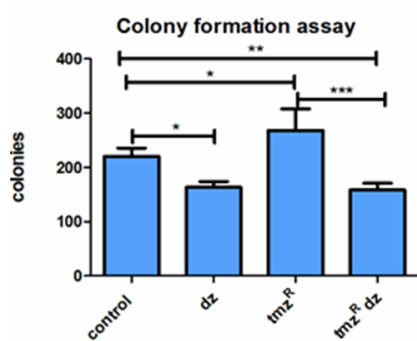


Figure 1C: Colony formation assay, showing a decrease in colony formation capacity of both cells treated with DZNeP: A172 cells (control), and A172-TMZ-R cells (temozolomide resistant, TMZR).

DZNeP decreases cell migration in control and TMZ-resistant glioblastoma cell lines

The scratching assay or wound healing assay allows a comparison of cell migration in different cell conditions by observing the process of wound closing. This experiment showed a decreased migration capacity in cells treated with DZNeP, both control and TMZ-resistant cell lines (Figure 1D). As in the colony formation assay, the TMZ-resistant cell line seems to be more tumorigenic than the control cell line. The wound is closed at 30 h in the resistant cells, while the control cells have not closed it at that time, which shows a more rapid migration of the TMZ-resistant cells.

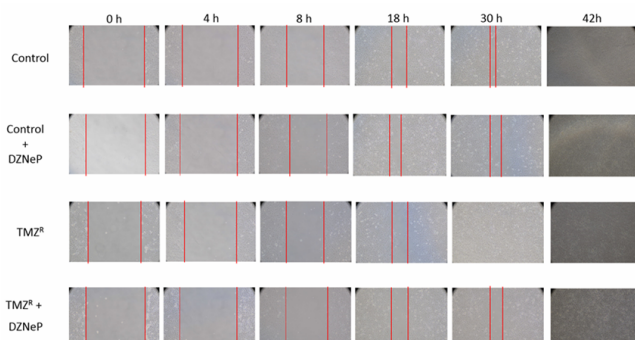


Figure 1D: Cell migration assay, showing a decrease in migration capacity of both cells after 30 h treatment with DZNeP: A172 cells (control), and A172-TMZ-R cells (temozolomide resistant, TMZR).

EZH2 and TGFβ1 pathways are downregulated in glioblastoma cells treated with DZNeP

After 72 h of DZNeP treatment in control and TMZ-resistant cells, gene expression of components of EZH2 and TGFβ1 pathways was analyzed by qRT-PCR. GAPDH was used as a housekeeping gene, but differences in its expression between the different samples were found. Therefore 4 possible housekeeping genes were analyzed in the samples: 2 metabolic genes (GAPDH and HPRT) and 2 structural genes (β-tubulin and β-actin) (Figure 1S, supplementary information). β-actin

was finally chosen as our housekeeping gene, as it had low CTs and the expression was homogeneous among samples.

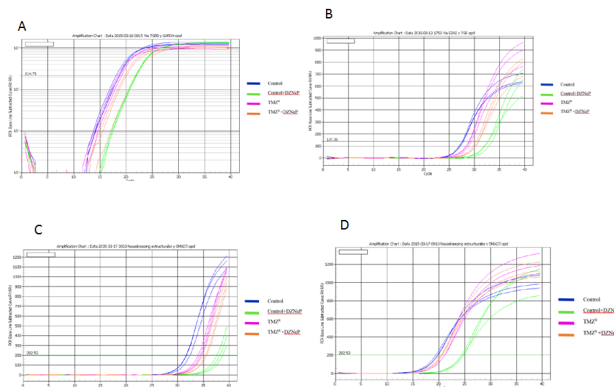


Figure 1S: A: GAPDH expression in control, control+DZNeP, TMZR, TMZR+DZNeP analyzed by qRT-PCR. B: HPRT expression in control, control+DZNeP, TMZR, TMZR+DZNeP analyzed by qRT-PCR. C: β-tubulin expression in control, control+DZNeP, TMZR, TMZR+DZNeP analyzed by qRT-PCR. D: β-actin expression in control, control+DZNeP, TMZR, TMZR+DZNeP analyzed by qRT-PCR.

The main target of DZNeP is EZH2. Results showed a decreased expression of EZH2 in both control and TMZ-resistant cells treated with DZNeP (Figure 2). Besides, E-cadherin and TIMP3, which are normally downregulated by EZH2, showed an increased expression in cells treated with DZNeP. However, the increase of these two genes was only significant in control cells, and not in the TMZ-resistant cells.

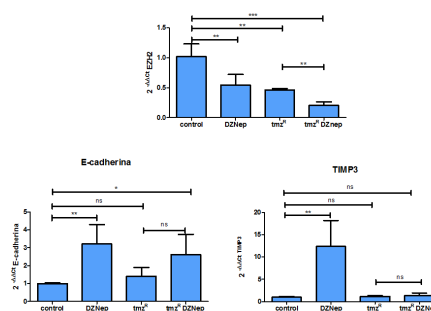


Figure 2: qRT-PCR results show that DZNeP decreases the expression of EZH2 and increases the expression of its targets E-cadherin and TIMP3 in both control and TMZ resistant cells. However, the increase of these two genes was only significant in control cells (A172), and not in A172-TMZ-R cells (temozolomide resistant, tmzR).

Previous studies found a relationship between EZH2 and TGFβ1 pathways [8], both overexpressed in cancer. Gene expression analysis by qRT-PCR suggested that TGFβ1 pathway is downregulated in cells treated with DZNeP, supporting that relationship (Figure 3). The expression of receptors involved in this pathway (TGFβR1 and TGFβR2) was decreased and the expression of inhibitors of this pathway (BAMBI, SMAD6 and SMAD7) was increased. Surprisingly,

the expression of TGFβ2 and TGFβ3 ligands was increased. This increase in the expression of ligands could be a response to the downregulation of the pathway (decreased receptors and increased inhibitors). However the increase of ligands cannot affect downstream steps in the pathway, as the number of receptors is decreased.

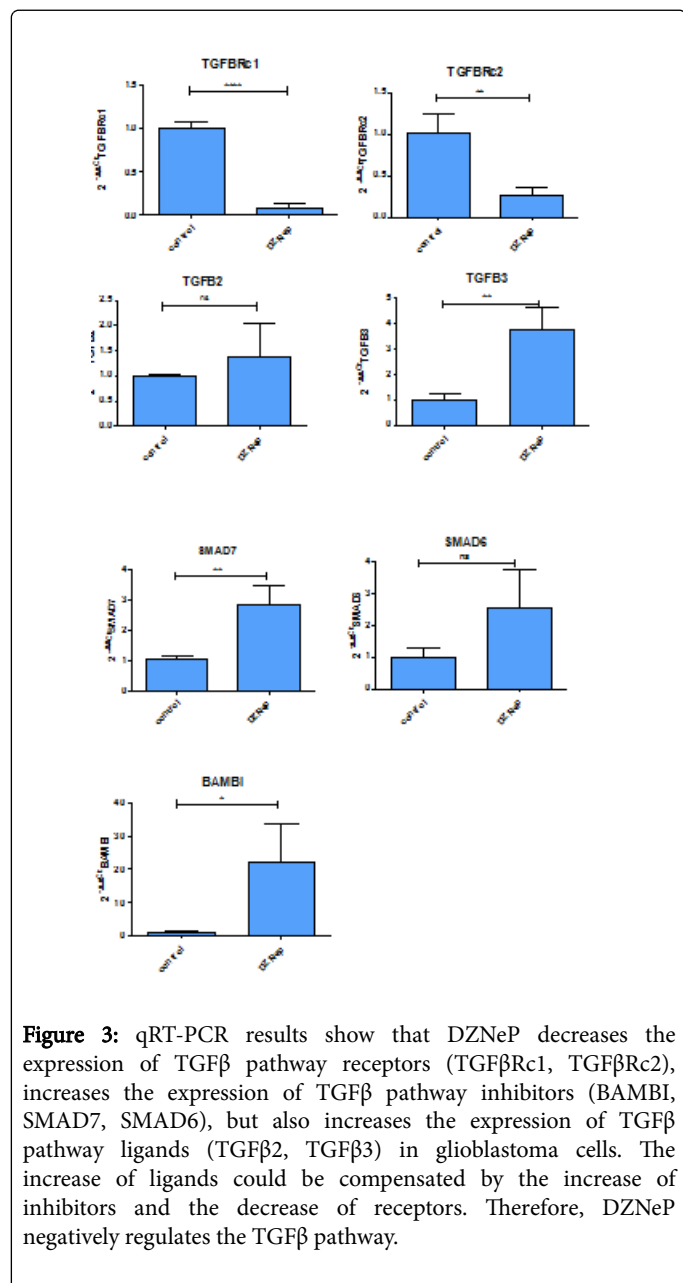


Figure 3: qRT-PCR results show that DZNeP decreases the expression of TGFβ pathway receptors (TGFβRc1, TGFβRc2), increases the expression of TGFβ pathway inhibitors (BAMBI, SMAD7, SMAD6), but also increases the expression of TGFβ pathway ligands (TGFβ2, TGFβ3) in glioblastoma cells. The increase of ligands could be compensated by the increase of inhibitors and the decrease of receptors. Therefore, DZNeP negatively regulates the TGFβ pathway.

In order to support the results obtained by qRT-PCR about the TGFβ1 pathway, protein expression of SMAD2 and PSMAD2 was analyzed by Western blot (Figure 4A). The ratio between PSMAD2/SMAD2 decreased in cells treated with DZNeP (Figure 4B). This result supports the possible therapeutic effect of this drug against the TGFβ pathway, as PSMAD2 is a negative prognostic marker in glioblastoma.

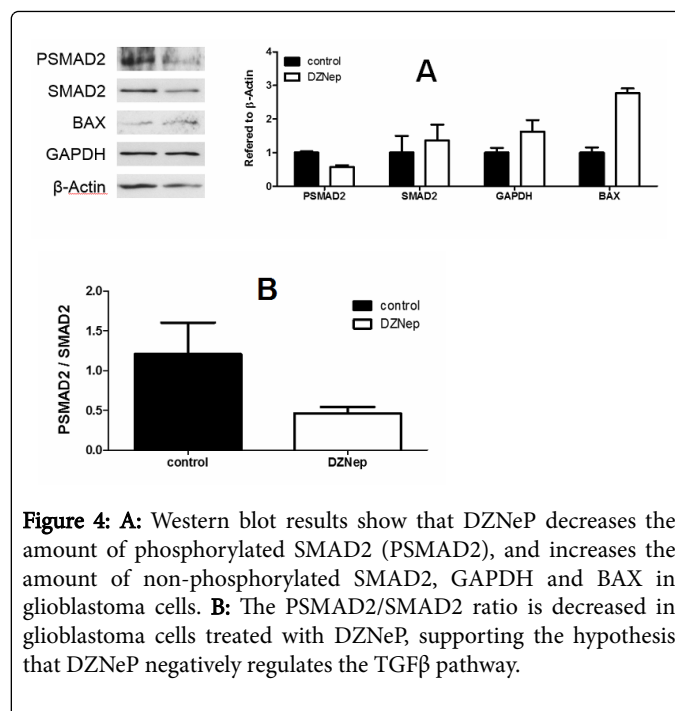


Figure 4: **A:** Western blot results show that DZNeP decreases the amount of phosphorylated SMAD2 (PSMAD2), and increases the amount of non-phosphorylated SMAD2, GAPDH and BAX in glioblastoma cells. **B:** The PSMAD2/SMAD2 ratio is decreased in glioblastoma cells treated with DZNeP, supporting the hypothesis that DZNeP negatively regulates the TGFβ pathway.

Besides, the housekeeping proteins GAPDH and β-actin were analyzed by Western blot, confirming the data obtained by qRT-PCR: β-actin is a better housekeeping gene in this experiment. Finally BAX protein was analyzed. This protein is related to apoptotic processes and it was increased in the cells treated with DZNeP, suggesting that these cells could be suffering apoptosis due to the treatment.

Discussion

Currently, TMZ resistance constitutes a big challenge in the treatment of glioblastoma, which is the most common form of malignant brain tumor in adults. Therefore, we tested a drug which has been assayed against several types of tumors with successful results: DZNeP. For this purpose we used the A172 glioblastoma cell line and we performed cellular and gene expression studies on control A172 glioblastoma cells, and on A172-TMZ-resistant glioblastoma cells.

Cellular studies

The purpose of these studies is to analyze the tumorigenic characteristics (proliferation, adhesion, colony formation and migration) of glioblastoma cells in 4 different conditions: control A172, control A172 treated with DZNeP, A172-TMZ-resistant, and A172-TMZ-resistant treated with DZNeP. The proliferation assay suggested that the TMZ-resistant and the control cells respond in a similar way to DZNeP. However, the results of the adhesion assay were not as clear. Even though it shows a decrease in the adhesion capacity in treated cells, a study with more time periods should be done to confirm these results.

Both migration and colony formation assays suggest that TMZ-resistant cells have a more tumorigenic behavior than control cells, as results indicate that resistant cells have a higher migration capacity and also a higher colony formation capacity. However, DZNeP had an effect over both cell types, causing a decrease in migration and colony formation capacity. This decrease is greater in TMZ-resistant cells.

Gene expression studies

The target of DZNeP is the EZH2 histone methyltransferase, which is overexpressed in cancer cells, decreasing the expression of genes involved in processes such as angiogenesis and metastasis inhibition. To confirm the effect of DZNeP on its target, the expression of EZH2 and two of its target genes (E-cadherin and TIMP3) was analyzed by qRT-PCR in control and TMZ-resistant cells with and without DZNeP treatment. The results showed a significant decrease on the expression of EZH2 in both treated control and treated resistant cells. They also present an increase in the expression of E-cadherin and TIMP3 in both cell types, which are usually downregulated in cancer, by the overexpression of EZH2. However, the statistical analysis determined that only the increase caused in control cells is significant.

This analysis suggests that DZNeP is effective on control cells, but its effectiveness on TMZ-resistant cells is not as clear. To confirm these results, more genes related to this pathway should be analyzed, for instance VASH1 which is related to angiogenesis, or FOXC1, related to metastasis. It would also be interesting to analyze the expression of micro RNAs such as miR-101 which is a physiological inhibitor of EZH2 and it is inhibited itself when EZH2 is overexpressed.

Previous studies suggested that the resistance to TMZ is due to the expression of MGMT caused by the lack of methylation on its promoter. Therefore it would be interesting to analyze and compare the methylation of MGMT promoter in control and TMZ-resistant cells and also in cells treated with DZNeP, to check if this treatment has any effect over the resistance to TMZ itself. Epigenetic processes like promoter methylation and histone methylation are closely related, so it could be helpful to analyze this relationship.

When analyzing the housekeeping genes in the different samples, it was found that the control cells treated with DZNeP had a lower expression of both, structural and metabolic genes. This could mean that the treatment caused apoptosis in these cells, leading to changes in the structure and metabolic pathways. In order to prove this, a protein related to apoptosis (BAX) was measured by Western blot in control cells with and without treatment. BAX levels were higher in cells treated with DZNeP, which supports the hypothetical apoptosis process in these cells. Besides, Western blot was also used to analyze the levels of GAPDH and SMAD2/phosphorylated SMAD2. GAPDH is one of the metabolic housekeeping genes analyzed by qRT-PCR, and the results showed higher levels of GAPDH protein in treated cells, which means the metabolism is affected in these cells, possibly by apoptosis.

SMAD2 is a transcription factor related to the TGF β pathway, which is phosphorylated when this pathway is upregulated. Phosphorylated SMAD2 has been proposed as a negative prognostic marker. The amount of PSMAD2 compared to the amount of SMAD2, is smaller in the cells treated with DZNeP. This supports the results obtained by qRT-PCR showing a decrease on the TGF β pathway.

Gene expression and protein studies suggested that DZNeP has a negative effect on EZH2 and TGF β pathways, both upregulated in cancer. Therefore, DZNeP seems to be a good alternative in the treatment of glioblastoma as it affects 2 pathways which are highly important in the development of this tumor. However, more studies should be done to confirm these results. For instance, all the experiments in this work were performed over samples treated for 72 h with the drug, which only allows us to see the effect at that time of exposure. It would be highly interesting to study gene expression with a kinetic assay, exposing cells to the drug for different amounts of time

(10, 20, 30, 40, 50, 60, 70, 80 h for instance). With this experiment it would be possible to analyze the expression of the genes of interest through time of treatment. Nevertheless, tumors inside the organism could react differently than cultured cells, as they are influenced by the environment, and many factors could affect the target pathways of DZNeP. Therefore, *in vivo* experiments should be performed testing the drug in regular tumors and also TMZ resistant tumors, to prove whether DZNeP could be useful for patients with TMZ-resistant glioblastomas.

As a conclusion and having in mind that further research should be done on this topic, we propose that inhibition of EZH2 might be considered as a therapeutic strategy against glioblastoma. After treatment with DZNeP (an EZH2 inhibitor) cell proliferation, cell adhesion, colony formation, and cell migration were inhibited *in vitro*, both in glioblastoma cells, and in TMZ-resistant glioblastoma cells. At the level of EZH2 target gene expression, DZNeP decreased EZH2 expression, and increased the expression of its target genes (E-cadherin and TIMP3), which might probably contribute to inhibiting the development of a cancer metastatic phenotype. Finally, DZNeP negatively regulated the TGF β pathway.

Acknowledgements

This research was supported in part by grants from Fundación Universitaria de Navarra, Pamplona, and Fondo de Investigación Sanitaria, Madrid: PI13/00055 (JAR), and PI13/00800 (BM).

References

1. Veliz I, Loo Y, Castillo O, Karachaliou N, Nigro O, et al. (2015) Advances and challenges in the molecular biology and treatment of glioblastoma-is there any hope for the future? *Ann Transl Med* 3: 7.
2. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, et al. (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17: 98-110.
3. Fan TY, Wang H, Xiang P, Liu YW, Li HZ, et al. (2014) Inhibition of EZH2 reverses chemotherapeutic drug TMZ chemosensitivity in glioblastoma. *Int J Clin Exp Pathol* 7: 6662-6670.
4. Lo HW (2010) EGFR-targeted therapy in malignant glioma: novel aspects and mechanisms of drug resistance. *Curr Mol Pharmacol* 3: 37-52.
5. Quick A, Patel D, Hadziahmetovic M, Chakravarti A, Mehta M (2010) Current therapeutic paradigms in glioblastoma. *Rev Recent Clin Trials* 5: 14-27.
6. Network CGAR (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455: 1061-1068.
7. Gall Troselj K, Novak Kujundzic R, Ugarkovic D (2016) Polycomb repressive complex's evolutionary conserved function: the role of EZH2 status and cellular background. *Clin Epigenetics* 8: 55.
8. Rao ZY, Cai MY, Yang GF, He LR, Mai SJ, et al. (2010) EZH2 supports ovarian carcinoma cell invasion and/or metastasis via regulation of TGF-beta1 and is a predictor of outcome in ovarian carcinoma patients. *Carcinogenesis* 31: 1576-1583.
9. Li K, Liu C, Zhou B, Bi L, Huang H, et al. (2013) Role of EZH2 in the growth of prostate cancer stem cells isolated from LNCaP cells. *Int J Mol Sci* 14: 11981-11993.
10. Crea F, Fornaro L, Bocci G, Sun L, Farrar WL, et al. (2012) EZH2 inhibition: targeting the crossroad of tumor invasion and angiogenesis. *Cancer Metastasis Rev* 31: 753-761.
11. Simon JA, Lange CA (2008) Roles of the EZH2 histone methyltransferase in cancer epigenetics. *Mutat Res* 647: 21-29.
12. Wang GG, Konze KD, Tao J (2015) Polycomb genes, miRNA, and their deregulation in B-cell malignancies. *Blood* 125: 1217-1225.

13. Smits M, Nilsson J, Mir SE, van der Stoop PM, Hulleman E, et al. (2010) miR-101 is down-regulated in glioblastoma resulting in EZH2-induced proliferation, migration, and angiogenesis. *Oncotarget* 1: 710-720.
14. Choi EJ, Cho BJ, Lee DJ, Hwang YH, Chun SH, et al. (2014) Enhanced cytotoxic effect of radiation and temozolomide in malignant glioma cells: targeting PI3K-AKT-mTOR signaling, HSP90 and histone deacetylases. *BMC Cancer* 14: 17.
15. Kim YZ (2014) Altered histone modifications in gliomas. *Brain Tumor Res Treat* 2: 7-21.
16. Joseph JV, Balasubramanian V, Walenkamp A, Kruyt FA (2013) TGF- β^2 as a therapeutic target in high grade gliomas - promises and challenges. *Biochem Pharmacol* 85: 478-485.
17. Massagué J (2008) TGFbeta in Cancer. *Cell* 134: 215-230.
18. Yan X, Liu Z, Chen Y (2009) Regulation of TGF-beta signaling by Smad7. *Acta Biochim Biophys Sin (Shanghai)* 41: 263-272.
19. Yan X, Lin Z, Chen F, Zhao X, Chen H, et al. (2009) Human BAMBI cooperates with Smad7 to inhibit transforming growth factor-beta signaling. *J Biol Chem* 284: 30097-30104.
20. Bruna A, Darken RS, Rojo F, Ocana A, Penuelas S, et al. (2007) High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. *Cancer Cell* 11: 147-160.
21. Gatza CE, Oh SY, Blobe GC (2010) Roles for the type III TGF-beta receptor in human cancer. *Cell Signal* 22: 1163-1174.