Title: Vismodegib Improved Therapy of Medulloblastoma by Targeting Sonic Hedgehog Signaling Pathway on DAOY Medulloblastoma Cell Line

Running Title: Vismodegib Effects on Medulloblastoma DAOY Cells

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Abstract

Introduction: Targeting SMO has been a remarkable strategy in Shh-dependent cancers, especially medulloblastoma. In this manner, GDC-0449 or vismodegib is used as a potent SMO inhibitor with mild toxicity and high affinity. Thus, the main purpose of the present study was to investigate the anti-medulloblastoma efficacy of vismodegib on the DAOY medulloblastoma cell line.

Methods: Human DAOY medulloblastoma cells were cultured in DMEM.50, 80, 100, and 150μM doses of vismodegib were used to treat cells. MTT, Scratch, and trypan blue assay, and also real-time polymerase chain reaction (RT-PCR) and immunofluorescence studies were performed, 24 and 48 hours post-treatment.

Results: MTT and trypan blue assay showed a significant difference in viability, between the control group and treatment groups. Results of the scratch assay showed that in the control group, the cells managed to repair the lesion, while the scratch was disintegrated in higher doses of vismodegib. The expression of SMO, Gli1, and MYCN genes, as the main components of the SHH signaling pathway, had a significant reduction in the vismodegib-treated groups compared to the control group. Also a notable rise in the activation of metastasis-promoting genes (Bax and p53) while a reduction in metastasis-inhibiting gene (Bcl2), was observed.

Conclusion: The results of the current study confirm that vismodegib is a potent inhibitor of the Shh pathway and could be used as a drug in combination with new therapeutic methods, in order to treat medulloblastoma.

Keywords: Vismodegib, Drug therapy, Medulloblastoma, Neoplasm, Toxicity

1. Introduction

Medulloblastoma is the most common childhood brain tumor, involving the cerebellum, with an extreme level of invasion (Bleil, Bizzi, Bedin, de Oliveira, & Antunes, 2019; Gendreau et al., 2020; McKinney, 2004; Waszak et al., 2020). The prognosis of medulloblastoma depends on different criteria. If the tumor is removed completely by surgery, there is no metastasis, and no tumor cells are visible in the CSF, the prognosis is usually good (McKinney, 2004; Szalontay & Khakoo, 2020). . Molecularly, 4 medulloblastoma subgroups are known to this date, which differ in mutation type and clinical symptoms (Northcott et al., 2011).Sonic hedgehog (Shh) subgroup engages two groups of infants (0 to 3 years old), and young adults (over 16 years old), is found in nearly 30% of cases, and involves cerebellar hemispheres. Both sexes are equally involved and its survival rate is about 40%. Shh is a signaling pathway important in organogenesis of almost all mammalian organs. It is widely inactive in adults(Schroeder & Gururangan, 2014).

The main components of the hh pathway include Shh, Ihh, and Dhh ligands, a negative regulatory receptor (Patched- PTCH), a positive regulator (Smoothened- SMO), and transcription factors of glioma-related oncogenes (GLI1, GLI2, and GLI3) (Carballo, Honorato, & de Lopes, 2018; Taylor et al., 2012). In brief, activation of SMO begins a cascade leading to Gli1 expression. The activated Gli1 is then transferred to the nucleus and induces Shh-pathway proteins (Lospinoso Severini et al., 2020).Recent studies have also proved that the Shh system plays a key role in the biology, differentiation, self-renewing, and tumorigenesis of cancer stem cells. It seems that overactivity of this pathway is the cause of resistance of cancer stem cells to chemotherapy (Mazumdar et al., 2011; Taylor et al., 2012). Overactivity of the Shh pathway is observed in many human cancers. In order to find new targeted treatments, inhibition of this signaling pathway has been a remarkable strategy for the

treatment of Shh-dependent cancers (Crotty et al., 2021; Doussouki et al., 2019; Menyhárt & Győrffy, 2020).

As SMO plays an important role in regulating the Shh pathway, targeting this protein has been a remarkable strategy in the treatment of Shh-dependent cancers, especially medulloblastoma (Lee, Zhao, & Ingham, 2016). Cyclopamine is a natural product derived from the Corn lilies plant, which is not suitable because of low solubility. Vismodegib and sonidegib are other SMO inhibitors that block the transmission of SMO to the cilium. GDC-0449 or Vismodegib is a Shh signaling (SMO) inhibitor and the first drug to be approved by the FDA for targeted SMO inhibition. The toxicity of this drug is generally mild (Sekulic et al., 2012; Singh, Fu, Srivastava, & Shankar, 2011) andIt is able to bind to SMO with a high affinity and effectively inhibit Shh-Gli signaling (Sekulic et al., 2012) MYCN is an oncogene responsible for the proliferation of the cerebellar granule cell precursors, which is the most considerable cause of medulloblastoma (Schwalbe et al., 2022). Its high expression represents poor prognosis, resistance to treatment, and metastasis (Shrestha, Morcavallo, Gorrini, & Chesler, 2021) . So, evaluating MYCN and its regulators seems to be promising in medulloblastoma treatment methods.

Despite advances in treatment modalities, the prognosis of medulloblastoma remains poor, especially in children with recurrent disease. In recent years, there have been significant advances in the understanding of the molecular biology of medulloblastoma, leading to the development of novel targeted therapies, but further studies are needed to determine the safety and efficacy of these treatmentsand improve the quality of life. Thus, the main purpose of the present study was to investigate the anti-apoptotic efficacy of Vismodegib on the DAOY medulloblastoma cell line.

2. Materials and methods

2.1. DAOY cell culture

Human DAOY medulloblastoma cell line was prepared from Pasteur Institute (Tehran, Iran), and cultured in DMEM (Dulbecco's Modified Eagle's Medium) high glucose with FBS 10% and penicillin-streptomycin 1% (at 37°C, 5 % CO2, and humidity of 95%). After about 3-5 days (confluency of 80%), cell passage was performed and incubation occurred for 24 hours. 50, 80, 100, and 150μM doses of vismodegib were used to treat cells. MTT, Scratch, and trypan blue assay, Real-time polymerase chain reaction(RT-PCR), and immunofluorescence studies were performed 24 and 48 hours post-treatment.

2.2. Vismodegib preparation

First, vismodegib (sc-396759/Santacruz, 10 mg powder), was purchased. The powder was dissolved using 1 ml DMSO as solvent. At this stage, 20 μL of the dissolved drug was diluted with 2.3 ml of DMEM and finally, vismodegib was obtained with a concentration of 200μM. Then, each dose of 50, 80, 100, and 150μM was prepared by dilution of 200μM solution.

2.3. MTT assay

5×10^3 DAOY cells were added in each well of the 96-well plate and 24 hrs of incubation (at 37°C, 5 % CO2, and humidity of 95%) was performed. After 24 hours, in each column of the plate, (except the control group) 50, 80, 100, and 150 μM doses of vismodegib were added respectively. After 24 and 48 hours of incubation, vismodegib and the culture medium were removed and 150-200 μL of MTT was replaced and incubated for 3 hours. After this time, MTT was removed and 150-200 μL of DMSO was added to each well. After the formation of formazan crystals, absorption of the samples was measured by an ELISA reader at 570 nm (Gorgich et al., 2022).

2.4. Scratch assay

Scratch assay isused to evaluate the rate of cell proliferation, and tissue repair. In order to perform this test, a scratch was created at the bottom of the 6-well plate, using the tip of the sampler (Figure 2). DAOY cells were seeded and incubated 24 hours before creating the scratch. In the next step, 50, 80, 100, and 150 μM doses of vismodegib were added to each well, respectively. Also, one of the wells was considered as control and did not receive any drugs. In order to evaluate the rate of proliferation and recovery, images of all wells were captured at 0, 24, and 48 hours after scratch. In the last step, images were evaluated and the distance between the two edges of the lesion was measured and compared in different groups(Figure 2) (Gorgich et al., 2022).

2.5. Trypan blue assay

In order to evaluate cell death, trypan blue staining was used. By analyzing the images taken from the samples, the percentage of dead cells in each dose of 50, 80, 100, and 150 was calculated and compared with the control group (figure 3).

2.6. RT-PCR assay

RT-PCR was applied to assess the expression of bcl2, Bax, p53, Gli1, SMO, and MYCN genes in DAOY cells, after treatment with vismodegib. Extraction of total RNA and synthesis of cDNA was performed using RNA extraction and cDNA synthesis kits, according to the manufacturer's instructions (FAVORGEN, Taiwan). In the last step, a Light cycler (Bioneer, Daejeon, South Korea) was used to perform RT-PCR. The relative gene expression was normalized to GAPDH and evaluated by the Ct method. The results were evaluated and the relative expression of bcl2, Bax, p53, Gli1, SMO, and MYCN genes was assessed as illustrated in Figure 4.

2.7. Immunofluorescence assay

Expression and localization of Gli1, SMO, and MYCN genes in DAOY medulloblastoma cells, were assessed using mouse anti-Gli1, anti-SMO, and anti-MYCN primary antibodies (Anti-Smoothened antibody (ab236465), Anti-n-Myc/MYCN antibody [NCM II 100] (ab16898), Anti-Gli1 antibody [HL247]), and Alexa flour568 conjugated secondary antibody (ab150113)). Nuclei were also labeled using bisbenzidine (DAPI). Final evaluation of samples was performed using a fluorescence microscope (Nikon Instruments Inc., NY, and USA). The stages are listed as follows: 1. Preparation of DAOY cells. 2. Washing with PBS, twice. 3. Fixing cells with 4% formaldehyde (200 microliters of paraformaldehyde in PBS), for 15 minutes at room temperature and under the hood. 4. Removal of fixative and washing with PBS twice, for 5 minutes. 5. Immersing in 100 µl of triton x 0.5 to 0.1%, diluted in PBS, for 15 minutes. 6. Removal of Triton x and washing with PBS twice, for 5 minutes. 7. Immersing in NGS 10% (100 µl in 1ml of PBS) for 6 minutes, at room temperature. 8. Removal of NGS, adding primary antibody diluted in PBS and incubation at 60 ºc, overnight. 9. Re-washing with PBS three times, 5 minutes each time. 10. Adding secondary antibody (3 µl in 200 µl of PBS, in the dark). 11. Washing with PBS, three times. 12. Adding DAPI (1 µg/ml of PBS) for 5 minutes, in the dark.

2.8. Statistical analysis

The normality of the data was checked using the Kolmogorov-Smirnov test. Statistical analysis of the data was performed using a One-way ANOVA test and post hoc analysis. Comparative charts were evaluated using Prism (version 9.1.2). P value less than 0.05 was reported to be significant. IBM SPSS software version 22 was used to analyze data.

3. Results

3.1. MTT assay

MTT assay results 24 hours after treatment showed that the viability of DAOY cells in doses of 50, 80, 100, and 150μM of Vismodegib had a significant difference compared to the control group. There was also a significant difference between doses 150, and 50 μM. MTT results 48 hours after treatment showed that the viability of DAOY cells in doses of 50, 80, 100, and 150μM had a significant difference with the control group. There was also a significant difference between doses of 100and 150 with 50μM, and the difference between 150 and 80μM was also significant (Figure 1).

3.2. Scratch assay

Analysis of images from scratch test, 0, 24, and 48 hours after the lesion, showed that in the control group, the cells managed to repair the lesion by maintaining their proliferation ability, while in the treatment groups, as the dose of vismodegib increased, the ability to repair the lesion decreased and due to cell disintegration, the scratch was not visible in higher doses (Figure 2).

3.3. Trypan blue assay

Analysis of images from trypan blue staining revealed that in both 24 and 48 hours post-treatment, the difference in cell death between the control group and 50μM dose was not significant. The difference between doses of 80,100 and 150μM was significant with both control and 50μM. and the difference between 100 and 150μM with 80μM was also significant. Moreover there was a significant difference between doses of 100 and 150μM (Figure 3).

3.4. RT-PCR assay

The expression of SMO, Gli1, and MYCN was significantly different between the control group and the treatment groups. There was also a significant difference in their expression level, between the G1 and G2 groups (24 and 48 hours after treatment with vismodegib, respectively). (Figure 4). The expression of bcl2 was significantly different between the control group and the treatment groups. There was also a significant difference in its expression level, between the G1 and G2 groups. The expression of Bax was significantly different between the control group and the treatment groups. There was also a significant difference in its expression level, between the G1 and G2 groups. The expression of p53 gene was not significantly different between the control group and the G1 group, while there was a significant difference between the G2 and the control group. On the other hand, there was no significant difference in the expression level of this gene, between the G1 and G2 groups (Figure 4).

3.5. Immunofluorescence assay results

Fluorescence evaluations represented that in the vismodegib-treated DAOY cells, the morphology of the nuclei was different compared to the control group, in a way that they became condensed and blebbed , which could be considered as pre-apoptotic changes (Figure 5). Using fluorescence microscopy, it was also understood that the expression of Gli1, SMO, and MYCN genes was reduced in treatment groups, compared to the control, as depicted in Figure 5.

4. Discussion

GDC-0449 or Vismodegib is a potent Shh inhibitor that binds to the SMO receptor, with high specificity and affinity. It is the first FDA-approved drug to be used in the treatment of Shh-cancers, with very low toxicity (Sekulic et al., 2012; Singh et al., 2011). Evidence suggests that Vismodegib effectively inhibits SMO, leading to reduced tumor growth in medulloblastoma,

lung cancer, pancreatic cancer, and leukemia (Carpenter & Ray, 2019). In a 2019 study, it was found that Vismodegib is able to inhibit cell proliferation in glioblastoma (Bureta et al., 2019). In 2020, Vismodegib was used in refractory prostate cancer. In this study, it was observed that vismodegib can affect apoptosis, cell proliferation, and epithelial-mesenchymal transformation in prostate cancer cells, by inhibiting Shh signaling (Ishii et al., 2020). In a 2020 study, the use of Vismodegib + itraconazole on oral Squamous Cell Carcinoma (OSCC), led to a decrease in cell viability and also morphological changes and apoptosis (Freitas et al., 2020). In 2021, Hwang et al, successfully improved the survival of medulloblastoma-suffering mice, using poly nanoparticle (2-oxazoline) containing Vismodegib (Hwang et al., 2021). Despite significant advances in the treatment of medulloblastoma in recent years, there is still much work to be done, to improve survival rates. The treatment of medulloblastoma depends on various factors such as: the size and location of the tumor, the age, and the overall health condition of the patient. In order to reduce the side effects and preserve the quality of life, a multidisciplinary approach is required. Continued research on the biology of medulloblastoma and the novel treatment modalities are essential in this manner. In this study, MTT, trypan blue, and scratch assays showed that vismodegib is able to affect cell growth in the DAOY medulloblastoma cell line, by reducing cell viability. In order to effective apoptosis, p53, and bax genes have to operate properly (Ramadan, Shawkey, Rabeh, & Abdellatif, 2019). In this study, vismodegib-treated groups represented a high expression of p53 and Bax, compared to the control group. On the other hand, a significant decrease in the expression of Bcl2 (anti-apoptotic) was found in the treatment groups, compared to the control (GAPDH was considered as internal control). Shh signaling pathway includes a wide range of proteins and effectors. SMO is a trans-membrane protein and a member of the G protein-coupled receptor family that plays a vital role in morphogenesis and cellular activity. Activation of SMO leads to a cascade, resulting in the activation of Gli1, as a downstream effector (Lospinoso Severini et al., 2020; Schulte &

Bryja, 2007). MYCN is an oncogene responsible for the proliferation of the cerebellar granule cell precursors, as the most considerable cause of medulloblastoma. The level of MYCN could determine the physiological and clinical consequences of medulloblastoma, in a way that its high expression represents poor prognosis, resistance to treatment, and metastasis. So, evaluating MYCN and its regulators seems to be promising in medulloblastoma treatment methods (Schwalbe et al., 2022; Shrestha et al., 2021). In the current study, the expression of SMO, Gli1, and MYCN genes showed a significant reduction in the vismodegib-treated groups., Also due to the notable rise in the expression of metastasis- promoting genes (Bax and p53) while a reduction in metastasis-inhibiting gene (Bcl2), it could be concluded that vismodegib is able to inhibit shh pathway, and induce apoptosis in DAOY medulloblastoma cells, and regarding previous studies, it could be used as a combination therapy in Shh-medulloblastoma.

5. Conclusion

The results of the current study confirm that vismodegib is a potent inhibitor of the Shh pathway in DAOY cells, under in vitro conditions. Due to its low toxicity and high affinity, it could be used as a combination therapy in the treatment of medulloblastoma.

Ethical Considerations

Compliance with ethical guidelines

This study was conducted based on guidelines by considering Helsinki Declaration. This study was conducted under the supervision of Iran University of Medical Sciences (IUMS) Ethics Committee IR.IUMS.FMD.REC.1399.574.

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Author Contributions

Design and conceptualization: FM, MB, RSh, and MM. Data statistics and analysis: MB, MGh, and AR, EACG, ES, FU. Acquisition of data: MB, MGh, and AR, EACG, ES, FU, and FM. Writing of the manuscript: MB, MGh, and AR, EACG, ES, FU, RSh, MM. Supervision: FM, RSh, MM. All authors read, revised and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no competing financial or non-financial interests directly or indirectly related to the work submitted for publication.

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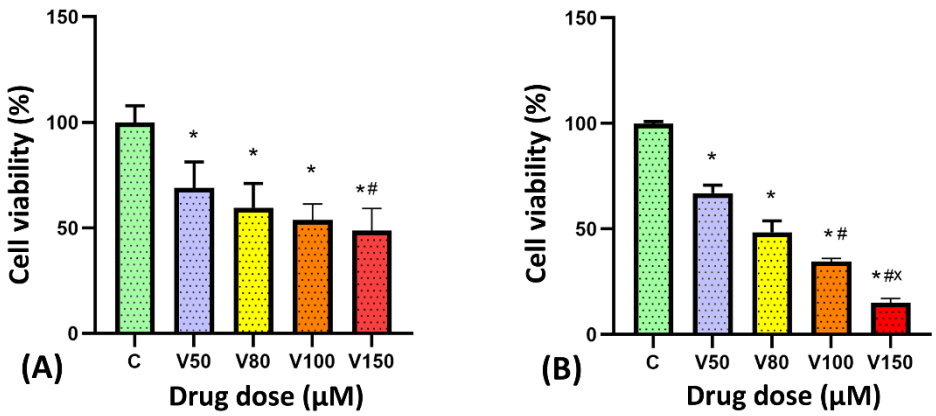

Figure 1. (A): cell viability 24hr post-treatment. **(B)**: cell viability 48hr post-treatment..

C: control. V50: Vismodegib 50µM. V80: Vismodegib 80µM. V100: Vismodegib 100µM. V150: Vismodegib 150µM

*: P <0.05 vs control

#: P <0.05 vs v50

x: P <0.05 vs v100

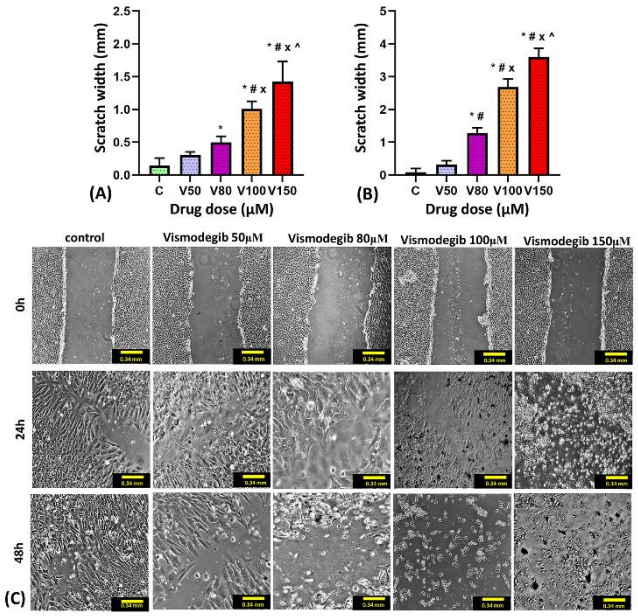

Figure 2. (A): scratch width 24hr post-treatment. **(B)**: scratch width 48hr post-treatment. **(C)**: images of 0, 24 and 48 hours after scratch.

C: control. V50: Vismodegib 50µM. V80: vismodegib 80µM. V100: Vismodegib 100µM. V150: Vismodegib 150µM

*: P <0.05 vs control

#: P <0.05 vs v50

x: P <0.05 vs v80

^: P <0.05 vs v100

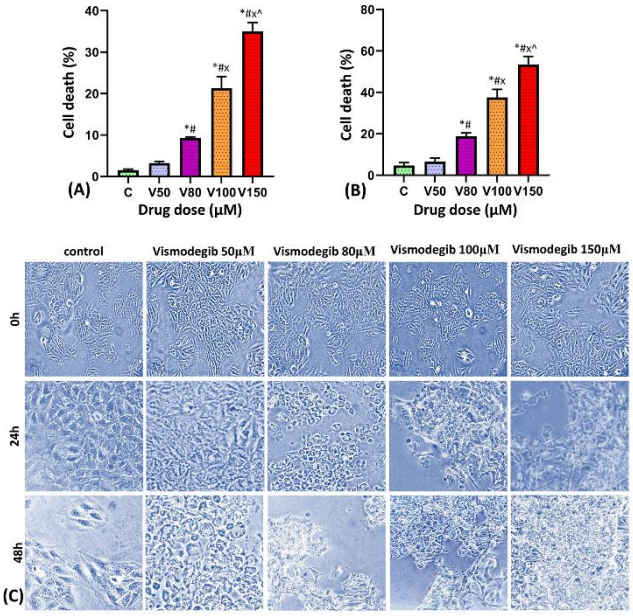

Figure 3. (A): cell death 24hr post-treatment. **(B)**: cell death 48hr post-treatment. **(C)**: images of 0, 24 and 48 hours after treatment with vismodegib.

(C): control. V50: Vismodegib 50µM. V80: vismodegib 80µM. V100: Vismodegib 100µM. V150: Vismodegib 150µM

*: P <0.05 vs control

#: P <0.05 vs v50

x: P <0.05 vs v80

^: P <0.05 vs v100

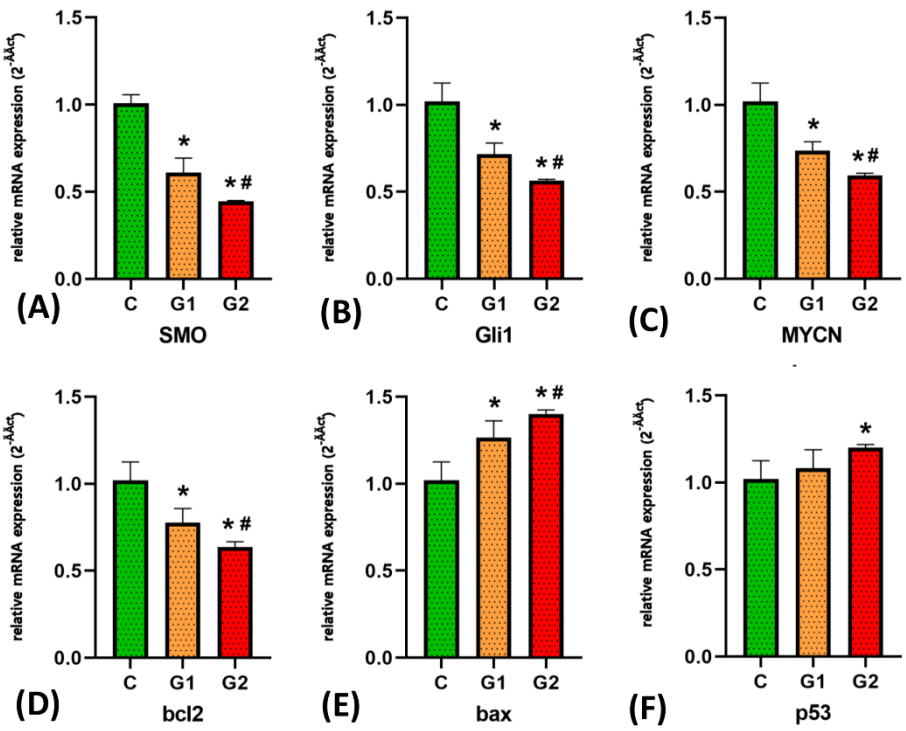

Figure 4. Relative gene expression of bcl2, Bax, p53, Gli1, SMO, and MYCN genes in DAOY cells, after treatment with vismodegib. Light cycler (Bioneer, Daejeon, South Korea) was used to perform RT-PCR. The relative gene expression was normalized to GAPDH and evaluated by the Ct method.

C = control

G1 = 24 h after treatment with vismodegib.

G2 = 48 h after treatment with vismodegib

*: P <0.05 vs control

#: P <0.05 vs G1

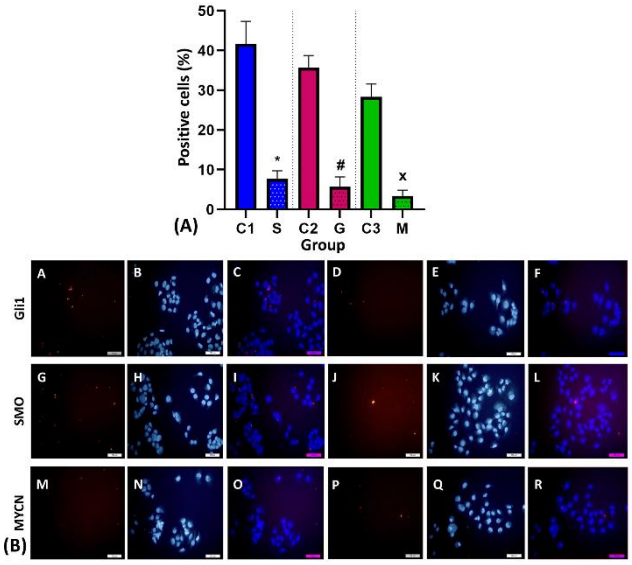

Figure 5. Expression and localization of Gli1, SMO and MYCN genes in DAOY medulloblastoma cells, was assessed using mouse anti-Gli1, anti-SMO and anti-MYCN primary antibodies (Anti-Smoothened antibody (ab236465), Anti-n-Myc/MYCN antibody [NCM II 100] (ab16898), Anti-Gli1 antibody [HL247]), and Alexa flour568 conjugated secondary antibody (ab150113)). Nuclei were also labelled using bisbenzidine (DAPI). Final evaluation of samples was performed using fluorescence microscope (Nikon Instruments Inc., NY, and USA). GAPDH was considered as internal control.

(A) percentage of positive cells

S: Smo, G: Gli1, M: Mycn

C1: Smo control, C2: Gli1 control, C3: Mycn control

*: P <0.05 vs C1

#: P <0.05 vs C2

x: P <0.05 vs C3

(B) Expression and localization of Gli1, SMO and MYCN genes in DAOY medulloblastoma cells

A-C = Gli1 control; D-F = Gli1 after treatment with vimodegib

G-I = SMO control; J-L = SMO after treatment with vimodegib

M-O = MYCN control; P-R = MYCN after treatment with vimodegib

Figure 6. a. ic50 of vismodegib, 24hr post treatment (calculated as: 134.21); b. ic50 of vismodegib, 48hr post treatment (calculated as: 78.06)