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Research Paper

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

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ABSTRACT

Introduction: Targeting smoothened (SMO) has been a remarkable strategy for treating sonic hedgehog (Shh)-dependent cancers, especially medulloblastoma. GDC-0449, also known as vismodegib, is a potent SMO inhibitor with mild toxicity and high affinity. Thus, this study aimed to investigate the anti-medulloblastoma efficacy of vismodegib in the DAOY medulloblastoma cell line.

Methods: Human DAOY medulloblastoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). 50, 80, 100, and 150 µM doses of vismodegib were used to treat cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), scratch, and trypan blue assays, as well as real-time polymerase chain reaction (RT-PCR) and immunofluorescence studies, were performed 24 and 48 h post-treatment.

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

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

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Highlights

- Vismodegib is a potent inhibitor of SMO in the DAOY medulloblastoma cell line.
- Vismodegib can regulate cell viability and apoptosis in Shh-dependent cancer cells.
- Vismodegib could be considered as adjunct therapy for Shh medulloblastoma.

Plain Language Summary

Vismodegib is a medication that targets a signaling pathway known as the Sonic Hedgehog (Shh) pathway. This pathway plays an important role in cell growth and development. In this study, we aimed to find out if vismodegib can affect a type of brain cancer, called medulloblastoma. We used human DAOY medulloblastoma cells line in the lab. The cells were treated with different doses of vismodegib. Then, we performed various tests to see the effects of the drug on the cells. Vismodegib reduced the viability of DAOY cells significantly. The scratch assay results showed that the cells that did not receive Vismodegib were able to repair the wound, but in those received higher doses of Vismodegib, the scratch was not repaired properly. This finding shows that the drug inhibits cell migration and regeneration. Vismodegib managed to reduce the expression of *SMO*, *Gli1*, and *MYCN*. These genes are crucial for Shh-cancer cell growth. It also increased the activation of *Bax* and *p53* genes which promote cancer, while reduced *Bcl2* gene, which inhibits cancer. This study confirms that vismodegib is a potent inhibitor of the Shh signaling pathway and it can be used as a promising treatment for Shh medulloblastoma.

1. Introduction

Medulloblastoma is the most common childhood brain tumor, typically involving the cerebellum, with a high level of invasion (Bleil et al., 2019; Gendreau et al., 2020; McKinney, 2004; Waszak et al., 2020). The prognosis of medulloblastoma depends on different criteria. If the tumor is completely removed by surgery, with no metastasis, and no tumor cells visible in the cerebrospinal fluid (CSF), the prognosis is usually good (McKinney, 2004; Szalontay & Khakoo, 2020). To date, four medulloblastoma subgroups are known to differ in mutation type and clinical symptoms (Northcott et al., 2011). The sonic hedgehog (Shh) subgroup engages two groups of infants (0 to 3 years old) and young adults (>16 years old) and is found in nearly 30% of cases, involving the cerebellar hemispheres. Both sexes are equally involved, and its survival rate is approximately 40%. Shh is a signalling pathway crucial in the 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; the negative regulatory receptor patched (PTCH); the positive regulator smoothened (SMO); and transcription factors of glioma-related oncogenes (*GLI1*, *GLI2*, and *GLI3*) (Carballo et al., 2018; Taylor et al., 2012). In brief, the activation of SMO initiates a cascade leading to *Gli1* expression. Activated *Gli1* is then transferred to the nucleus, where it induces Shh pathway proteins (Lospinoso Severini et al., 2020). Recent studies have also demonstrated that the Shh system plays a crucial role in the biology, differentiation, self-renewal, and tumorigenesis of cancer stem cells. It seems that overactivity of this pathway is the primary cause of resistance in cancer stem cells to chemotherapy (Mazumdar et al., 2011; Taylor et al., 2012). Overactivity of the Shh pathway has been observed in many human cancers. To identify new targeted treatments, inhibiting this signalling pathway has been a remarkable strategy for treating Shh-dependent cancers (Crotty et al., 2021; Doussouki et al., 2019; Menyhárt & Győrffy, 2020).

As SMO plays a crucial role in regulating the Shh pathway, targeting this protein has been a remarkable strategy for treating Shh-dependent cancers, especially medulloblastoma (Lee et al., 2016). Cyclopamine is a natural product derived from corn lilies, which is unsuitable due to its low solubility. vismodegib and sonidegib are other SMO inhibitors that block the transmission of SMO to cilia. GDC-0449, or vismodegib, is a Shh signalling (SMO) inhibitor and the first drug approved by the Food and Drug Administration (FDA) for targeted SMO inhibition. The toxicity of this drug is generally mild (Sekulic et al., 2012; Singh et al., 2011), and it can

bind to SMO with high affinity, effectively inhibiting Shh-Gli signalling (Sekulic et al., 2012). *MYCN* is an oncogene responsible for the proliferation of cerebellar granule cell precursors, which is the most significant cause of medulloblastoma (Schwalbe et al., 2022). High expression is associated with poor prognosis, treatment resistance, and metastasis (Shrestha et al., 2021). Therefore, evaluating *MYCN* and its regulators appears promising for medulloblastoma treatment.

Despite advances in treatment modalities, the prognosis of medulloblastoma remains poor, especially in children with recurrent disease. In recent years, significant advances have been made in understanding the molecular biology of medulloblastoma, leading to the development of novel targeted therapies. However, further studies are needed to determine the safety and efficacy of these treatments and improve the quality of life. Thus, this study aimed to investigate the anti-apoptotic efficacy of vismodegib on the DAOY medulloblastoma cell line.

2. Materials and Methods

DAOY cell culture

Human DAOY medulloblastoma cell line was prepared from Pasteur Institute (Tehran Province, Iran) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with fasting blood sugar (FBS) 10% and penicillin-streptomycin 1% (at 37 ˚C, 5% carbon dioxide [CO_2], and humidity of 95%). After approximately 3-5 days (confluency of 80%), the cells were passaged, and incubated for 24 h. Vismodegib was used at doses of 50, 80, 100, and 150 µM to treat the cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), scratch, and trypan blue assays, real-time polymerase chain reaction (RT-PCR), and immunofluorescence studies were performed 24 and 48 h post-treatment.

Vismodegib preparation

First, vismodegib (sc-396759/Santacruz, 10 mg powder) was purchased. The powder was dissolved in 1 mL of dimethyl sulfoxide (DMSO) as the solvent. At this stage, 20 µL of the dissolved drug was diluted with 2.3 mL of DMEM, and, vismodegib was obtained at a concentration of 200 µM. Each dose of 50, 80, 100, and 150 µM was prepared by diluting the 200 µM solution.

MTT assay

DAOY cells were added to each well of the 96-well plate, incubation was performed for 24 h (at 37 ˚C, 5% carbon dioxide [CO_2], and humidity of 95%). After 24 hours, doses of 50, 80, 100, and 150 µM vismodegib were added to each column of the plate (except the control group). 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, the absorption of the samples was measured using an ELISA reader at 570 nm (Gorgich et al., 2022).

Scratch assay

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

Trypan blue assay

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 (50, 80, 100, and 150) was calculated and compared with the control group (Figure 3).

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. Total ribonucleic acid (RNA) was extracted and complementary DNA (cDNA) was synthesized using RNA extraction and cDNA synthesis kits, respectively, according to the manufacturer's instructions (FAVORGEN, Taiwan). In the final step, a light cycler (Bioneer, Daejeon, South Korea) was used to perform RT-PCR. Relative gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and evaluated using 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.

Immunofluorescence assay

The 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 labelled with bisbenzidine (DAPI). Final evaluation of samples was performed using a fluorescence microscope (Nikon Instruments Inc., NY, USA). The stages are as follows: 1. Preparation of DAOY cells. 2. Wash twice with phosphate-buffered saline (PBS). 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 next-generation sequencing (NGS) 10% (100 µL in 1 mL of PBS) for 6 minutes, at room temperature. 8. Removal of NGS, adding primary antibody diluted in PBS and incubation at 60°C overnight. 9. Re-washing with PBS three times for 5 minutes each. 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.

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 analysis of variance (ANOVA) test, followed by post hoc analysis. Comparative charts were evaluated using GraphPad Prism software, version 9.1.2. $P<0.05$ was considered significant. IBM SPSS software, version 22 was used to analyze data.

3. Results

MTT assay

MTT assay results 24 hours after treatment showed that the viability of DAOY cells at 50, 80, 100, and 150 µM of vismodegib differed significantly from that of the control group. Also, a significant difference was observed between doses 150, and 50 µM. MTT results 48 hours after treatment showed that the viability of DAOY cells at 50, 80, 100, and 150 µM differed significantly from that of the control group. A significant difference was observed between 100 and 150 with 50 µM, and between 150 and 80 µM (Figure 1).

Scratch assay

Analysis of images from the scratch test at 0, 24, and 48 h after the lesion showed that in the control group, the cells managed to repair the lesion by maintaining their proliferative ability. In contrast, 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).

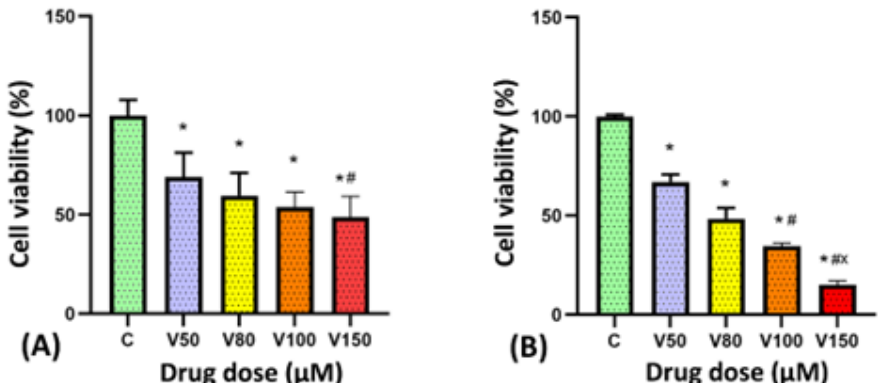

Figure 1. Cell viability in the control and treatment groups

A) Cell viability 24 h post-treatment, B) Cell viability 24 h post-treatment

Abbreviations: C: Control; V50: Vismodegib 50 µM; V80: Vismodegib 80 µM; V100: Vismodegib 100 µM; V150: Vismodegib 150 µM.

*P>0.05 vs control, #P>0.05 vs v50, xP>0.05 vs v100.

Trypan blue assay

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

RT-PCR assay

The expression levels of *SMO*, *Gli1*, and *MYCN* were significantly different between the control and treatment groups. Also, a significant difference was observed 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 and treatment groups. Also, a significant difference was observed in its expression level, between the G1 and G2 groups. The expression of *Bax* was significantly different between the control and treatment groups. A significant difference was observed in its expression level between the G1 and G2 groups. The expression of the *p53* gene was not significantly different between the control and G1 group, while a significant difference was observed between the G2 and control groups. On the other hand, no significant difference was observed in the expression level of this gene, between the G1 and G2 groups (Figure 4).

Immunofluorescence assay results

Fluorescence evaluations revealed that in vismodegib-treated DAOY cells, the morphology of the nuclei differed from that of the control group, with nuclei becoming condensed and blebbed, which could be considered pre-apoptotic changes (Figure 5). Fluorescence microscopy revealed that the expression of *Gli1*, *SMO*, and *MYCN* genes was reduced in the treatment groups compared to the control group (Figure 5).

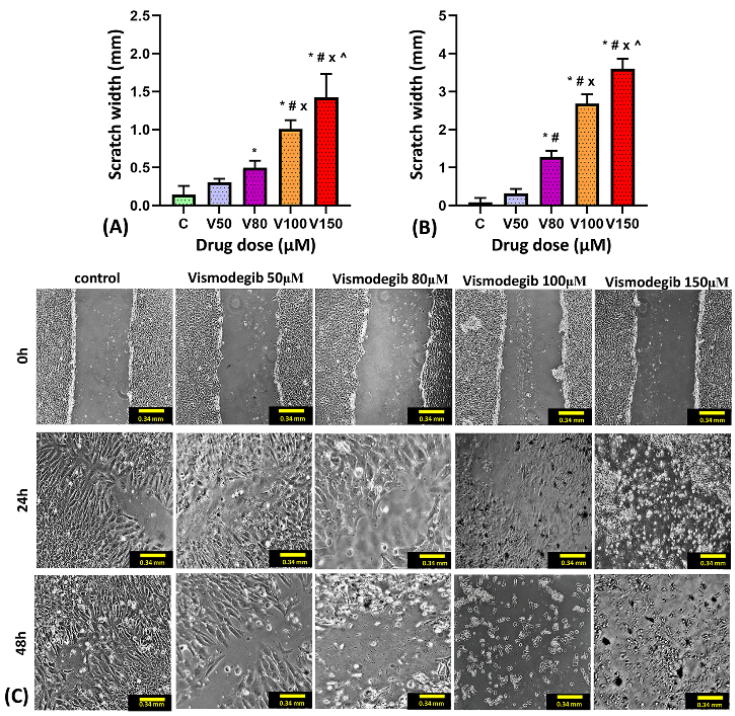

Figure 2. Scratch width in the control and treatment groups

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A) Scratch width 24 h post-treatment, B) Scratch width 48 h post-treatment, C) Images of 0, 24 and 48 h after scratch

Abbreviations: C: Control; V50: Vismodegib 50 µM; V80: Vismodegib 80 µM; V100: Vismodegib 100 µM; V150: Vismodegib 150 µM.

*P>0.05 vs control, #P>0.05 vs v50, xP>0.05 vs v80, ^P>0.05 vs v100.

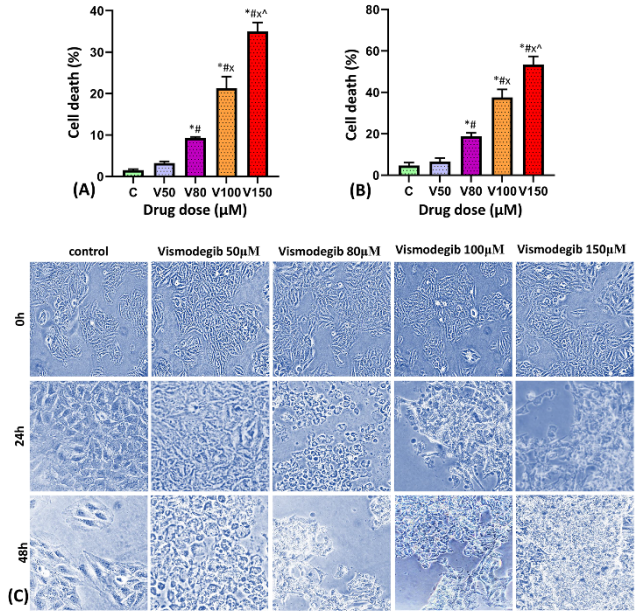

Figure 3. Cell death in the control and treatment groups

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A) Cell death 24 h post-treatment, B) Cell death 48 h post-treatment, C) Images of 0, 24 and 48 h after treatment with vismodegib

Abbreviations: C: Control; V50: Vismodegib 50 µM; V80: Vismodegib 80 µM; V100: Vismodegib 100 µM; V150: Vismodegib 150 µM.

*P>0.05 vs control, #P>0.05 vs v50, xP>0.05 vs v80, ^P>0.05 vs v100.

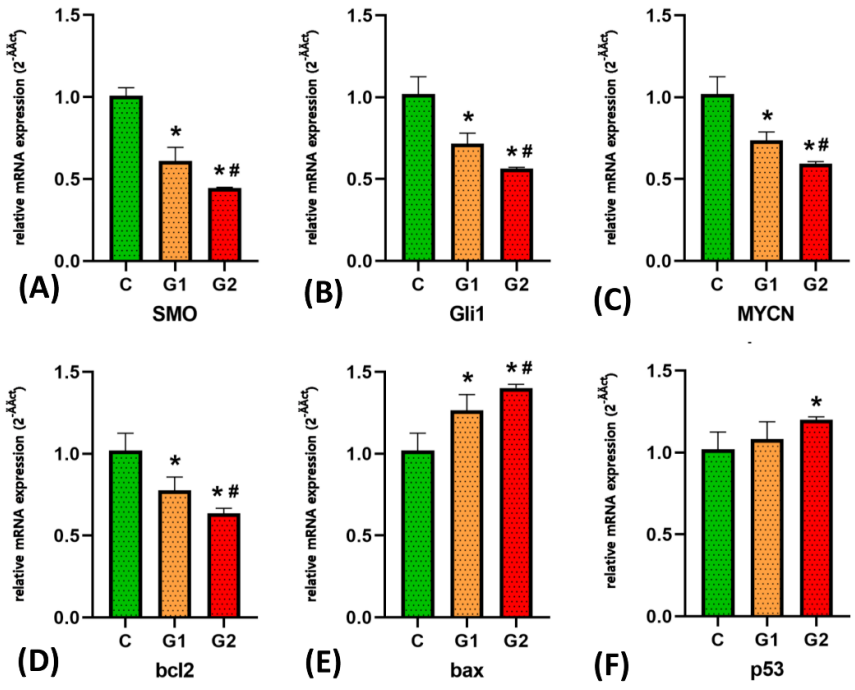

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Figure 4. Relative gene expression of *bcl2*, *Bax*, *p53*, *Gli1*, *SMO*, and *MYCN* genes in DAOY cells, after treatment with vismodegib

*P>0.05 vs control, #P>0.05 vs G1.

Note: 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.

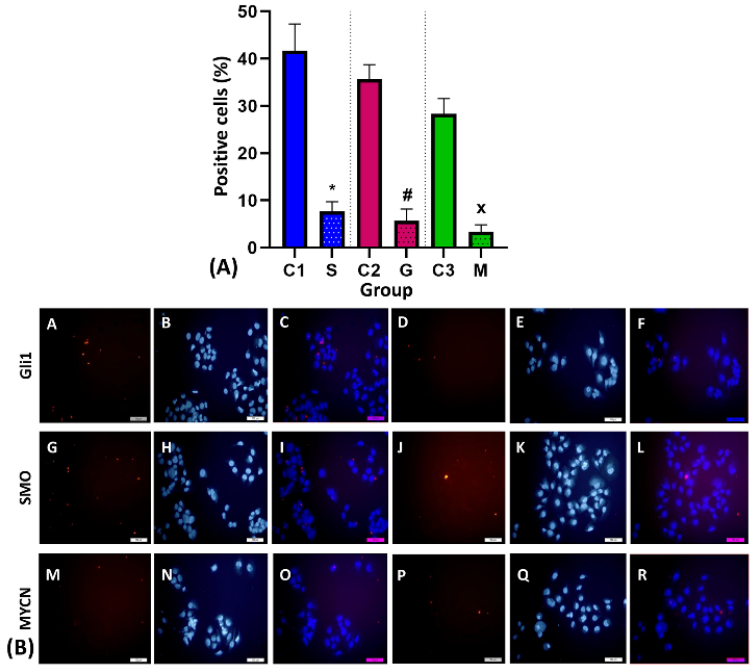

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Figure 5. Expression and localization of *Gli1*, *SMO* and *MYCN* genes in DAOY medulloblastoma cells assessed using mouse anti-Gli1, anti-SMO and anti-MYCN primary antibodies (ab236465, ab16898, HL247, and ab150113)

A) Percentage of positive cells, B) Expression and localization of *Gli1*, *SMO* and *MYCN* genes in DAOY medulloblastoma cells

Abbreviations: 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, xP>0.05 vs C3. Note: 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. Nuclei were also labelled using DAPI. Final evaluation of samples was performed using fluorescence microscope (Nikon Instruments Inc., NY, and USA). *GAPDH* was considered as internal control.

4. Discussion

GDC-0449 (vismodegib) is a potent Shh inhibitor that binds to the SMO receptor, with high specificity and affinity. It is the first FDA-approved drug 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). A 2019 study found that vismodegib inhibited cell proliferation in glioblastoma (Bureta et al., 2019). In 2020, vismodegib was used for 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 for treating oral squamous cell carcinoma resulted in decreased cell viability, 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 medulloblastoma treatment in recent years, considerable work remains to further improve survival rates. The treatment of medulloblastoma depends on various factors, such as the tumor size and location, patient age, and their overall health condition. A multidisciplinary approach is required to reduce side effects and preserve quality of life. Continued research on the biology of medulloblastoma and novel treatment modalities are essential. In this study, MTT, trypan blue, and scratch assays showed that vismodegib can affect cell growth in the DAOY medulloblastoma cell line by reducing cell viability. For effective apoptosis, the *p53* and *Bax* genes must function properly (Ramadan et al., 2019). In this study, the vismodegib-treated groups exhibited higher *p53* and *Bax* expression than the control group. In contrast, 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 triggers a cascade that activates Gli1 as a downstream effector (Lospinoso Severini et al., 2020; Schulte & Bryja, 2007). *MYCN* is an oncogene responsible for the proliferation of cerebellar granule cell precursors, which is the most considerable cause of medulloblastoma. The level of *MYCN* can determine the physiological and clinical consequences of medulloblastoma, as its high expression represents a poor prognosis, resistance to treatment, and metastasis. Therefore, evaluating *MYCN* and its regulators appears promising for 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*) and a reduction in the expression of the metastasis-inhibiting gene (*Bcl2*), it can be concluded that vismodegib can inhibit the Shh pathway and induce apoptosis in the DAOY medulloblastoma cells. In addition, based on previous studies, it can 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 can be used in combination therapy for the treatment of medulloblastoma.

Ethical Considerations

Compliance with ethical guidelines

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

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Authors' contributions

Conceptualization and study design: Fatemeh Moradi, Masume Behruzi, Ronak Shabani, and Mehdi Mehdizadeh; Data acquisition: Masume Behruzi, Mehrdad Ghorbanlou, Auob Rustamzadeh, Enam Alhagh Gorgich, Elham Seidkhani, Farnoosh Usefi, and Fatemeh Moradi; Data statistics and analysis: Masume Behruzi, Mehrdad Ghorbanlou, Auob Rustamzadeh, Enam Alhagh Gorgich, Elham Seidkhani, and Farnoosh Usefi; Writing the original draft: Masume Behruzi, Mehrdad Ghorbanlou, Auob Rustamzadeh, Enam Alhagh Gorgich, Elham Seidkhani, Farnoosh Usefi, Ronak Shabani, and Mehdi Mehdizadeh; Supervision: Fatemeh Moradi, Ronak Shabani, and Mehdi Mehdizadeh; Review, editing and, final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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