

Flavopiridol suppresses cell proliferation and migration and induces apoptotic cell death by inhibiting oncogenic FOXM1 signaling in IDH-wild type and -mutant GBM cells

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Article

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Abstract

Glioblastoma multiforme (GBM) remains one of the most challenging solid cancers to treat due to its highly aggressive and drug resistant nature. Flavopiridol is synthetic flavone that was recently approved by the FDA for the treatment of acute myeloid leukemia. Flavopiridol exhibits antiproliferative activity in several solid cancer cells and currently evaluated in clinical trials in several solid and hematological cancers. In this study, we investigated the molecular mechanisms underlying antiproliferative effects of Flavopiridol in GBM cell lines with wild type and mutant IDH1 (encoding isocitrate dehydrogenase 1). We found that Flavopiridol inhibits proliferation, colony formation, migration, and induces apoptosis in IDH1-wild type and IDH-mutant cells through inhibition of FOXM1 oncogenic signaling. Furthermore, flavopiridol treatment also inhibits of NF- κ B, mediators unfolded protein response (UPR) (GRP78, PERK, IRE1 α) and DNA repair enzyme PARP, which have been shown, be potential therapeutic targets by downregulating FOXM1 in GBM cells.

Our findings suggest for the first time that flavopiridol suppresses proliferation, survival and migration and induces apoptosis in IDH1-wild type and mutant GBM cells by targeting FOXM1 oncogenic signaling which also regulates NF- κ B, PARP, UPR response in GBM cells. Flavopiridol may be a potential novel therapeutic strategy in the treatment of patients IDH1-wild type and mutant GBM.

Introduction

Glioblastoma multiforme (GBM) the second leading cause of death from cancer in worldwide [1]. GBM, which originate from glial cells, is the most common and the most aggressive type of human brain tumors, constituting up to 81% of malignant brain tumors [2, 3]. Surgical resection, followed by chemo- and radiotherapy is currently the standard treatment for GBM in the clinic [4]. However, therapeutic efficiency of the standard therapy is low due to the invasive nature of glioblastoma and 90% of patients experience tumor recurrence within 6–9 months after initial treatment and an average survival of patients is about 15 months [4]. Therefore, it is of great significance to find effective therapeutic strategies and alternative compounds for the treatment of GBM.

GBM is a high grade glioma (grade IV) [4] and exome-sequencing studies identified that isocitrate dehydrogenase (IDH) 1 or 2 gene mutations was commonly detected in glioma tumors [5]. According to mutational status of IDH gene, in the 2016 update of the WHO, GBM is classified as (1) IDH1-wildtype and (2) IDH1-mutant GBM [6]. The current classification emphasized that most aggressive form of GBM is the one with wild type IDH1 [7, 8]. GBM tumors with wild type IDH represents 90% of GBMs, while IDH-mutant form is detected in 10% of GBMs [4, 9].

IDH mutations commonly occur in lower-grade glioma (i.e., tumors that had progressed from WHO grade II/III) and in secondary GBMs, whereas it's rarely observed in de novo or primary GBMs [9]. IDH enzymes normally catalyze the decarboxylation of isocitrate to generate α -ketoglutarate (α -KG), but mutant IDH enzymes loses normal enzymatic activity and produces 2- hydroxyglutarate (2-HG), which is an

oncometabolite [10, 11]. 2-HG inhibits α -KG-dependent enzymes that play crucial roles in gene regulation and DNA or histone demethylases [10, 11]. Epigenetic alterations induced via 2-HG in DNA and histones levels lead to malignant transformation by altering gene expression and blocking normal differentiation processes in Glioma cells [10, 11].

Flavopiridol is a semi-synthetic flavonoid isolated from an Indian plant, *Dysoxylum binectariferum* [12, 13]. Flavopiridol has been identified as an effective antitumor agent for several cancers. Even, flavopiridol has been tested in clinical trials [12, 14, 15], and was recently approved by the FDA in acute myeloid leukemia for its clinical activity [16, 17]. Flavopiridol has been shown to inhibit CDKs in G1/S or G2/M of cell cycle in hematological malignancies and solid tumors [12, 18–23]. Also, flavopiridol inhibits cell proliferation, migration and metastasis in anaplastic thyroid cancer, KRAS-mutant lung adenocarcinoma, and osteosarcoma cells [18, 21, 24]. Furthermore, flavopiridol induces endoplasmic reticulum (ER) stress and autophagy in chronic lymphocytic leukemia cells [25]. Flavopiridol inactivates glycogen phosphorylate and decrease glucose levels [26]. Furthermore, it was shown that flavopiridol has antiproliferative activity against GBM [27, 28] and down regulated expression of Akt, c-Myc, and cyclin-D HIF-1 α and GLUT1 expression in GBM cells [28]. Flavopiridol enhances the cytotoxicity of temozolomide and radiation in GBM cells [29, 30]. However, the exact mechanisms by which flavopiridol induced effects in GMB and IDH1 mutant GBM cells remains to be elucidated.

Forkhead box protein M1 (FOXM1) is an oncogenic transcription factor and plays a key role in cell cycle as master regulator of genes involved in cell cycle [31]. FOXM1 plays important roles in major hallmarks of cancer such as angiogenesis, invasion, and increases apoptosis resistance, autophagy and tumorigenesis in cancer cells [31–35]. FOXM1 is overexpressed in a wide range of cancers including GBM. Elevated expression of FOXM1 in GBM is strongly linked to aggressive phenotype, and it's expression associated with shorter survival and worse prognosis of GBM patients [36, 37]. Expression of mutant IDH1 gene is induced by oncogenic FOXM1. Therefore, FOXM1 was proposed as a potential therapeutic target for GBM with IDH1 mutations [29, 38]. However, currently there is no FDA-approved FOXM1 inhibitor for clinical translation.

In the current study we investigated the effects of flavopiridol on FOXM1 signaling IDH1-wild type and mutant GBM cells. Our findings demonstrated for first time that flavopiridol inhibits cell proliferation, colony formation and migration of IDH1-wild type and IDH1-mutant GBM cells by suppressing FOXM1 signaling. Our data suggest that oncogenic FOXM1 signaling promotes NF- κ B and mediators of unfolded protein response (UPR), including, GRP78, PERK, and IRE1 α and DNA-repair enzyme PARP and flavopiridol inhibits these pathways through suppressing FOXM1 in GBM cells. Since the use of flavopiridol can inhibit FOXM1 involving multiple oncogenic signaling, it may be tested in in vivo studies to determine the therapeutic efficacy and may be used in the treatment of GBM.

Materials And Methods

Cell lines, culture conditions, and reagents

Human IDH1-wild type U87-MG (cat# HTB-14) and IDH1-mutant-U87 (cat#HTB-14IG) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). U87-MG and IDH1-mutant-U87 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). Flavopiridol (Sigma-Aldrich, Cat# F3055.) were purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved distilled water to prepare stock solutions. This were then diluted with FBS-free medium before applying to cells.

Transfection with siRNA

Two different small interfering RNAs (FOXM1#1 siRNA, Cat# SASI_Hs01_00052108; FOXM1#2 siRNA, Cat# SASI_Hs01_00243977) targeting Forkhead box M1 (FOXM1) gene and non-silencing control siRNA (Cat# WD00909801) were purchased from Sigma-Aldrich. Exponentially growing U87-MG and IDH1-mutant-U87 GBM cells were plated 24h before transfection and transfected with two different FOXM1 siRNAs or control siRNA at a final concentration of 50 nM for 72h, using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Non-silencing control siRNA-transfected cells were used as negative controls [32–35]. After treatment, the cells were harvested and processed for further analysis

Cell viability and proliferation assays

Cell viability and proliferation were measured by MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI) as described previously [32, 34]. U87-MG and IDH1-mutant-U87 GBM cells were seeded in 96-well plates (1.20×10^5 cells/well) and treated with increasing doses of flavopiridol (5, 10, 25, 50, 100, 200, 300 and 400 nM) for 24h, 48h and 72h and incubated at 37°C. Furthermore, cells were seeded in 96-well plates (1.20×10^5 cells/well) transfected with a non-silencing control siRNA and FOXM1 siRNAs at a final concentration of 50 nM for 72h. After treatment with flovopiridol, solution containing MTS and phenazine methosulfate (20:1 v/v) was added to the cells. After 3 h of incubation at 37°C, the number of viable growing cells was estimated by measuring absorption at 490 nm, using Elisa Reader based on generation of formazan by the cells [32, 34]

Colony formation assays

To detect proliferation and colony formation of U87-MG and IDH1-mutant-U87 GBM cells treated with flovopiridol, we performed clonogenic assay as described previously [32, 34]. For this assay, cells were seeded in six-well plates (1.5×10^3 cells/ well); treated with increasing doses of Flavopiridol (5, 10, 15, 20, 30, 40, 60, 80, 100, 150 and 200 nM) and incubated at 37°C for 2 weeks to form colonies. Furthermore, cells were seeded in 6-well plates (1.5×10^3 cells/well) transfected with a non-silencing control siRNA and FOXM1 siRNAs at a final concentration of 50 nM for 72h, and grown for 2 weeks. Then, the cells were washed with PBS and stained with crystal violet, and visible colonies were counted.

Cell migration and motility assay

To detect migration and motility of U87-MG and IDH1-mutant-U87 GBM cells treated with flovopiridol, we performed wound healing assay [32]. For this assay, cells were seeded in six-well plates (1.5×10^5 cells/well) and treated with flovopiridol for 24h (300 and 400 nM), 48h (100 and 200 nM) and 72h (50 and 100 nM). Furthermore, cells were seeded in six-well plates (5×10^5 cells/well) and 24 h later were transfected with the control siRNA or FOXM1 siRNAs (50 nM). After incubation, each cell monolayer was carefully scratched using a 20- μ l sterile tip and cellular debris was removed by washing with medium, which was then replaced with fresh medium. Cells in the scratched area were imaged at 0 h, 24 h and 48 h using light microscopy, and the distance traveled by cells at the leading edge of the wound at each time point was measured. The results were expressed as average distance between the edges of the gap.

Assessment of nuclear morphological features

To observe changes in nuclear morphology of U87-MG and IDH1-mutant-U87 GBM cells treated with flovopiridol, we performed Hoechst 33258 staining. For Hoechst 33258 staining, cells were seeded in six-well plates (1×10^5 cells/well) and treated with flovopiridol for 24h (300 and 400 nM), 48h (100 and 200 nM) and 72h (50 and 100 nM). After treatment with flovopiridol, cells were harvested and washed with three times ice-cold PBS. Subsequently, 4% paraformaldehyde was used to fix the cells for 30 min. Then, 4% paraformaldehyde was removed and washed with three times ice-cold PBS. The cells were then stained with 200 μ l Hoechst 33258 (Sigma, 0.5 mg/mL) for 30 min in the dark. After stained, the Hoechst solution was then removed, and cells were washed with three times ice-cold PBS, followed by addition of fresh PBS. The changes in the nuclei of cells were evaluated with a fluorescence microscope using 320–350 nm filters (Eclipse Ti, Nikon). The morphological changes of nucleus were observed as condensed and fragmented nuclei [39].

Apoptotic cell death analysis

Apoptosis was assessed by annexin V/PI staining. For apoptosis staining, U87-MG and IDH1-mutant-U87 GBM cells were seeded in 96-well plates (1×10^4 cells/well) and treated with flovopiridol for 24h (300 nM) and 48h (100 and 200 nM). Furthermore, cells were seeded in six-well plates 96-well plates (1×10^4 cells/well) and 24 h later were transfected with the control siRNA or FOXM1 siRNAs (50 nM). After incubation, the supernatant was removed and cells were blocked with Fc-block for 5 min on ice. Then, cells were stained with BioLegend's FITC Annexin V Apoptosis Detection Kit with 7-AAD (cat#640922) and analyzed with FACSARIAIII (Becton Dickinson) [40].

Western Blot analysis

For western blot analysis, U87-MG and IDH1-mutant-U87 GBM cells (3.5×10^5) were seeded in T-25 culture flasks and treated with and treated with flavopiridol for 24h (300 nM), 48h (100 and 200 nM) and 72h (50 and 100 nM). Furthermore, U87-MG and IDH1-mutant-U87 GBM cells were transfected with siRNAs (50 nM) for 72 h. Then, the cells were collected, washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in a lysis buffer at 4°C. The protein concentrations were measured with a protein assay kit (DC kit; Bio-Rad, Hercules, CA). A total of 40 μ g of protein from each sample was separated by Sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis with a 4–20% gradient and

transferred to polyvinylidene difluoride membranes. The membranes were blocked with a blocking buffer (0.1 Triton X-100 with 5% dry milk in Tris-buffered saline–Tween 20 (TBS-T) for 60 min. After being washed with TBS-T, the membranes were probed with the following primary antibodies: FOXM1 (Cell Signaling, cat#5436S), NF- κ B (Abcam, cat# ab16502), PARP (Cell Signaling, cat# 9542S), GRP78 (Proteintech, cat# 11587-1-AP), PERK (Cell Signaling, cat# D11A8), IRE1 α (Cell Signaling, cat#14C10), and β -actin (Proteintech, cat# 60008-1-Ig). After being washed with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (Bio-Rad, #170–6515) or anti-mouse secondary antibody (Bio-Rad). β -actin was used as a loading control. All antibodies were diluted in TBS-T containing 5% dry milk. Chemiluminescence detection was performed with Clarity Western ECL Substrate (Biorad) and the blots were visualized with a ChemiDoc MP Imaging System (Biorad) and quantified with a densitometer using the ChemiDoc MP Imager application program (Biorad) [32–35].

Statistical analysis

All experiments were conducted at least in triplicate, and the results were summarized as means with standard deviations. Statistical significance was determined using the Student t test. p values less than 0.05 were considered statistically significant. GraphPad Prism program was used to evaluate the data and plot the graphs.

Results

Flavopiridol inhibits proliferation, colony formation and migration of IDH-wild type and IDH1-mutant GBM cells

To investigate effects of flavopiridol on IDH-wild type and IDH-mutant GBM cells proliferation, both U87-MG and IDH1-mutant-U87 GBM cells were treated with various concentrations of flavopiridol (5 to 400 nM) for 24, 48, and 72 hours. The MTS analysis revealed that treatment of cells with flavopiridol resulted in a dose-dependent inhibition of cell proliferation compared to untreated (NT) in both U87-MG cells and IDH1-mutant-U87 cells (Fig. 1, A-F). Although 24 hours of flavopiridol treatment exhibited modest effects on cell viability, 72 hours of treatments at even 100 nM of flavopiridol was effective in suppression cell proliferation in both cells (Fig. 1, C, F). We then investigated the effects of flavopiridol on colony formation of U87-MG cells and IDH1-mutant-U87 cells and found that Flavopiridol treatment at 150 and 200 nM led to a significant reduction in the number of colonies in U87-MG cells (Fig. 1, G, I) and IDH1-mutant-U87 cells (Fig. 1, H, J), respectively.

To identify the effects of flavopiridol on cell motility and migration, both IDH1-mutant-U87 cells and U87-MG cells were treated with flavopiridol (300 and 400 nM for 24; 100 or 200 nM for 48h; 50 or 100 nM for 72 h). As shown in Fig. 1K, N, while the untreated cells completely closed the wounded areas by migrating into the open areas, in flavopiridol treated cells significantly reduced cell migration as indicated by larger open areas with much less number of cells at 48 h and 72 h. Overall, these results suggested that flavopiridol decrease cell motility and migration in both IDH-wild type and IDH1-mutant GBM cells.

Flavopiridol Induces Apoptosis, Alters Nuclear Morphology, And Parp Expression Inhibits In And Idh1-mutant Gbm Cells

To determine if flavopiridol induces apoptosis we performed Annexin V assay in GBM cells. As shown in Fig. 2A, B, treatment of cells with flavopiridol (300 for 24; 100 and 200 nM for 48h) led to significant a dose- and time-dependent apoptosis in IDH-wild type and IDH1mutant-U87 cells. To evaluate the changes in cell nuclear morphology after treatment with flavopiridol in U87-MG cells and IDH1-mutant-U87 cells (300 and 400 nM for 24; 100 and 200 nM for 48h; 50 and 100 nM for 72 h) .We, subsequently, performed nuclear staining with Hoesct. The dye chromatin of apoptotic cells more brightly than chromatin of normal cells. As shown Fig. 2C-F, untreated cells showed a dull blue color indicating healthy and viable cells, while flavopiridol treated cells showed a population of cells which showed bright blue fluorescence with condensed or fragmented nuclei (the morphological changes of cell apoptosis).

Furthermore, we demonstrated that treatment with flavopiridol significantly reduced expression of PARP protein, which is involved in DNA repair and cell death, and plays an important role in GBM [41] (Fig. 3, A, D, H, K). Overall, these results suggested that promotes apoptotic cell death and suppresses PARP in both IDH1mutant-U87 cells and U87-MG cells.

Flavopiridol Suppresses Foxm1/nf-b And Upr Mediators' Expressions In Idh-wild Type And Idh1-mutant- Gbm Cells

FOXM1 is an oncogenic transcription factor that is highly overexpressed in GBM, and is associated with shorter overall patient survival [36]. Similarly, we found that protein expression level of FOXM1 highly expressed in IDH wild types and IDH1-mutant-U87 cells (Fig. 3, A, H). Thus, we first investigated the effect of flavopiridol on the expression level of FOXM1 in GBM cells. Flavopiridol treatment markedly reduced FOXM1 expression in a dose and time dependent manner in IDH wild type U87-MG cells (Fig. 3, A, B) and IDH1-mutant-U87 cells compared to the control cells (Fig. 3, H, I).

FOXM1 has been reported to function jointly with NF- κ B in target gene regulation [42]. Previously studies demonstrated that transcription factor NF- κ B and FOXM1 are hyper-activated in some cancers and play a critical role in development and progression of GBM [37, 43]. Also, NF- κ B is shown as molecular target in GBM, and is linked to poor prognosis. NF- κ B-induced genes have been shown to be promoting cell survival and proliferation of GBM cells [44]. Therefore, we investigated the effect of flavopiridol on NF- κ B. As expected, we found that flavopiridol significantly suppressed expression of NF- κ B dose- and time-dependent manner in both U87-MG (Fig. 3, A, C) and IDH-mutant-U87 (Fig. 3, H, J) cells compared to control cells.

Activation of NF- κ B has been shown to be associated with ER stress and unfolded protein response (UPR) [45–47]. GRP78, IRE1 α and PERK are known to control UPR, promoting cell proliferation, migration and invasion of GBM cells, and are associated with progression of GBM. [48]. Because FOXM1 promoted

GRP78 transcription [49] and activation of NF- κ B is modulated by UPR [47], we next investigated the effect of flavopiridol on the expression of UPR mediator's activation. Our results showed that flavopiridol treatment markedly decreased GRP78, PERK and IRE1 α expression in a dose and time dependent manner wild type U87-MG cells (Fig. 3, A, E-G).and IDH1-mutant-U87 (Fig. 3, H, L-N) cells compared to control cells.

Knockdown of FOXM1 inhibits colony formation and migration and induces apoptosis IDH-wild type and IDH1-mutant- GBM cells

To demonstrate if FOXM1 inhibition is the major molecular mechanism underlying flavopiridol-induced suppression of cell proliferation, we investigated the effects of FOXM1 knockdown on the colony formation of GBM cells. We found that knockdown of FOXM1 by specific siRNAs significantly inhibited colony formation in IDH wild type U87-MG cells (Fig. 4, A) and IDH1-mutant-U87 cells (Fig. 4, B) compared to control cells treated with control-siRNA.

To determine if flavopiridol-induced apoptosis is mediated by downregulation of FOXM1, we knocked down FOXM1 by siRNA and analyzed if FOXM1 inhibition induces apoptosis in IDH1-mutant-U87 cells and U87-MG cells. Cells were transfected with FOXM1 siRNAs (50 nM) and 72 h later stained with Annexin V and positive cells were quantified with Flow cytometry. As shown in (Fig. 4, C, D), FOXM1 inhibition induced significantly apoptosis in the GBM cells.

To identify the effects of FOXM1 on cell motility and migration, both IDH1-mutant-U87 cells and U87-MG cells were transfected with FOXM1 siRNAs (50 nM). As shown in (Fig. 4, E, F), while the treated cells with control siRNA completely closed the wounded areas by migrating into the open areas, in FOXM1siRNAs-treated GBM cells had significantly reduced cell migration as indicated by larger open areas with much less number of GBM cells.

Knockdown of FOXM1 suppressed expressions of NF- κ B and UPR mediators in IDH-wild type and IDH1-mutant- GBM cells

To confirm that flavopiridol inhibits NF- κ B, PARP and UPR activity through down regulation of FOXM1, we knocked down FOXM1 using specific siRNAs in two different GBM cells and evaluated expressions of FOXM1, NF- κ B, PARP, GRP78, IRE1 α and PERK by Western blot analysis. Knockdown of FOXM1 decreased expression of FOXM1, NF- κ B, PARP, GRP78, PERK and IRE1 α in wild-type IDH (Fig. 5, A-G) and IDH1-mutant-U87 cells compared to control cells (Fig. 5, H-N). Overall, these results indicated that FOXM1 regulates NF- κ B/PARP/UPR axis and its down regulation flavopiridol-induced effects in multiple signaling pathways and UPR-mediators.

Discussion

GBM is a highly aggressive malignant brain tumors but the prognosis of GBM remains very poor with a median survival of approximately 14 to 15 months from the diagnosis despite treatments with standard

therapies [4]. In recent years, some natural compounds such as flavopiridol have been reported to exhibit anti-proliferative activity in GBM cells [27; 28; 50–55]. However, exact mechanism of flavopiridol-induced effects remains to be elucidated in IDH-wild type and IDH-mutant GBM cells. Previous studies indicated that FOXM1 is overexpressed and regulates oncogenic signal pathways in GBM cells and plays an important role in cell proliferation, survival and migration [37, 38]. Our study showed for the first time that flavopiridol reduced cell proliferation, colony formation, migration, and led to nuclear fragmentation and induced apoptosis in both GBM cells through suppression of FOXM1 expression. Furthermore, our study provided the first evidence that FOXM1 is critical factor for GBM cell survival and its inhibition induced apoptosis in GBM cells, FOXM1 induces downstream NF- κ B signaling and mediators of UPR, including GRP78, PERK and IRE α and PARP. Moreover, we found that NF- κ B, UPR and PARP oncogenic signaling was significantly inhibited by flavopiridol-induced suppression of FOXM1 in both IDH-mutant and IDH-wild type GBM cells.

FOXM1 plays a critical role in the development and progression of GBM, and is associated with worse prognosis of patients [36–38]. Recent studies showed that FOXM1 are aberrantly activated in GBM cells and promoted multiple biological behaviors, including cell proliferation, cell cycle, metastasis, epithelial to mesenchymal transition, chemosensitivity and angiogenesis through regulating pathways such as MMP-2 and VEGF [56, 57], Rad51 [58], STAT3 [59], ADAM17/EGFR [60] and Wnt/ β -catenin [58]. We demonstrated that inhibition of FOXM1 expression by flavopiridol significantly suppressed GBM cells having different genetic profiles proliferation, colony formation, migration and induced apoptosis, and lead to reduce NF- κ B, UPR activity and PARP signaling, known as therapeutic target for GBM.

In cancer cells increased protein synthesis rates, more gene mutations and the stressful microenvironment such as hypoxia, hypoglycemia induces ER stress. ER stress leads to the activation of UPR which adaptive system. Thus, cancer cells use the UPR to survive harsh conditions rather than undergoing apoptosis. Therefore, UPR is activated in cancer cells [46, 61]. UPR is controlled by 78 kDa glucose-regulated protein (GRP78) and three different sensors, inositol-requiring enzyme 1 (IRE1 α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [45]. Several studies in glioma and other cancer cell lines have demonstrated that ER stress-associated proteins such as GRP78 have pro-survival roles. In GBM, upregulation of GRP78 has been linked to tumor grade, temozolomide resistance, and prognosis [62, 63]. Also, it has been reported that GBM tumor aggressiveness and chemoresistance correlates with elevated levels of GRP78 or IRE1 α [64]. In GBM mouse models IRE1 α has been reported to be induces angiogenesis, invasion and mesenchymal differentiation [64]. Furthermore, PERK also was found to stimulate GBM growth [65]. All of these results demonstrate that the ER stress and GRP78, IRE1 α and PERK, which are the major mediators regulating UPR, are involved in malignant characteristics of GBM such as cellular proliferation, migration, and invasion. Luo et al. [49] demonstrated that FOXM1 bind to promoter of GRP78 and increased GRP78 transcription in colorectal cancer.

NF- κ B is a transcription factor mediating inflammation, cell differentiation, proliferation and survival [43]. It promotes tumor progression by sustaining cell viability, inducing angiogenesis, metastasis, invasion, and regulating metabolic adaption and increased resistance to therapy in GBM (66–68). Hence, NF- κ B

has been proposed as a molecular target for GBM therapy [43]. Our studies indicated that flavopiridol inhibited NF- κ B expression in IDH-mutant and IDH-wild type GBM cells through inhibition of oncogenic FOXM1 signaling. Previous studies have demonstrated that FOXM1 regulates NF- κ B signaling, and NF- κ B activation was prevented inhibiting of FOXM1 [42, 66, 67]. Therefore, we expected to detect NF- κ B inhibition in GBM cells following flavopiridol and FOXM1 siRNA treatments. Other studies showed that flavopiridol treatment suppressed activation of NF- κ B in several cell types [68, 69] and in non-small cell lung carcinoma cell line [69] and human leukemia cells [70] support our findings.

UPR activity is significantly elevated in GBM compared to normal tissue, and it enhances cell survival, angiogenesis, metastatic capacity, drug resistance [71, 72]. Various stress conditions such as energy deprivation, hypoxia, and oxidative stress induce accumulation of misfolded or unfolded proteins as, consequence ER stress. Thereby, as a response ER stress, UPR pathway is activated to restore homeostasis, improve ER protein folding capacity and counteract the occurring damage in cells [61]. Some studies have shown that NF- κ B signaling is linked with UPR signaling involving PERK and IRE1 α in various cell types [45, 46, 73]. In GBM cells is UPR commonly up-regulated to promote cell survival under hypoxia, and therapy-induced stress conditions for escaping from cell death [71]. UPR is operated with three sensor proteins, PERK, IRE1- α , ATF-6 α . Because GRP78 is highly expressed in GBM and is correlated with increased malignancy and chemoresistance, it is strongly mediate signal transduction pathways inducing cell viability and metastatic ability in GBM. Therefore, GRP78 has been suggested as a cancer-specific target. We found that flavopiridol treatment markedly inhibited GRP78 expression in both IDH-wild types and IDH-mutant GBM cells. However, several studies have reported that UPR signaling involving PERK and IRE1 α can promote multi-oncogenic signal pathways, including NF- κ B signaling in various cell types [46, 47]. Thus, IRE1 α and PERK pathway rather than ATF6 pathway I part of the UPR has been proposed as promising a specific target for development of targeted therapies for GBM [63, 71]. Therefore, we also investigated effect of flavopiridol on PERK and IRE1 α expression and found that flavopiridol treatment markedly inhibited expression of PERK and IRE1 α in IDH-wild type and IDH-mutant GBM cells. These our results indicated that suppression with flavopiridol of UPR activation contributes to inhibition of cell proliferation, migration and the induction of cell death in GBM.

In addition, the presented study showed that flavopiridol treatment markedly leads to decreased expression of total PARP1 protein in IDH wild type and IDH mutant GBM cells. PARP plays roles in DNA repair. Thus, PARP inhibition causes break accumulation during DNA replication and induces apoptosis. It has been demonstrated that PARP inhibitors approved by the FDA for the treatment of various cancer the induce apoptosis [74, 75]. PARP plays a central role in GBM biology, and PARP1 expression level correlated positively with increasing tumor grade and poorer overall survival [41]. Therefore, acts of PARP inhibitors are recently being investigated to potentiate the effects of conventional therapies and overcome acquire resistance to standard therapies in GBM cells and patients [4, 41]. In the presented study, flavopiridol treatment led to morphological changes including condensation of chromatin and nuclear fragmentation, and induced apoptosis by inhibiting PARP expression in IDH wild type and IDH mutant GBM cells.

Overall, all of these findings suggest that among FOXM1, NF- κ B, UPR activity, and PARP is molecular interaction, and this interaction promotes proliferation, colony formation and resistance to apoptosis in both GBM cells. Although DH1 mutant GBM cells have metabolic and epigenetic reprogramming different from wild-type IDH1 cells depending on the IDH gene mutation [76], interestingly, our results showed that Flavopiridol has a similar effect on both GBM cells.

Due to crucial role of FOXM1 in development and progression of GBM [38, 58], targeting FOXM1 expression by flavopiridol may have significant clinical implications in controlling growth of IDH mutant GBM tumors and be an effective strategy in FOXM1 overexpressing GBM tumors.

Declarations

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Statements and Declarations

Consent for publication: This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosures and declarations: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper, and approved the final version of the manuscript being submitted. The results presented in this paper have not been published previously in whole or part.

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Author contributions: All authors analyzed the results and approved the final version of the manuscript. Zuhail Hamurcu conceptualized, coordinated, administered the study, performed experiments and wrote the paper. Bulent Ozpolat conceived, conceptualized, supervised the study and revised and edited the manuscript. Ahsen Guler, Venhar Cinar, Nursultan Nurdinov Serife Erdem helped performing the experiments. Halil Ulutabanca helped writing the paper. All authors read and approved the final manuscript.

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Figures

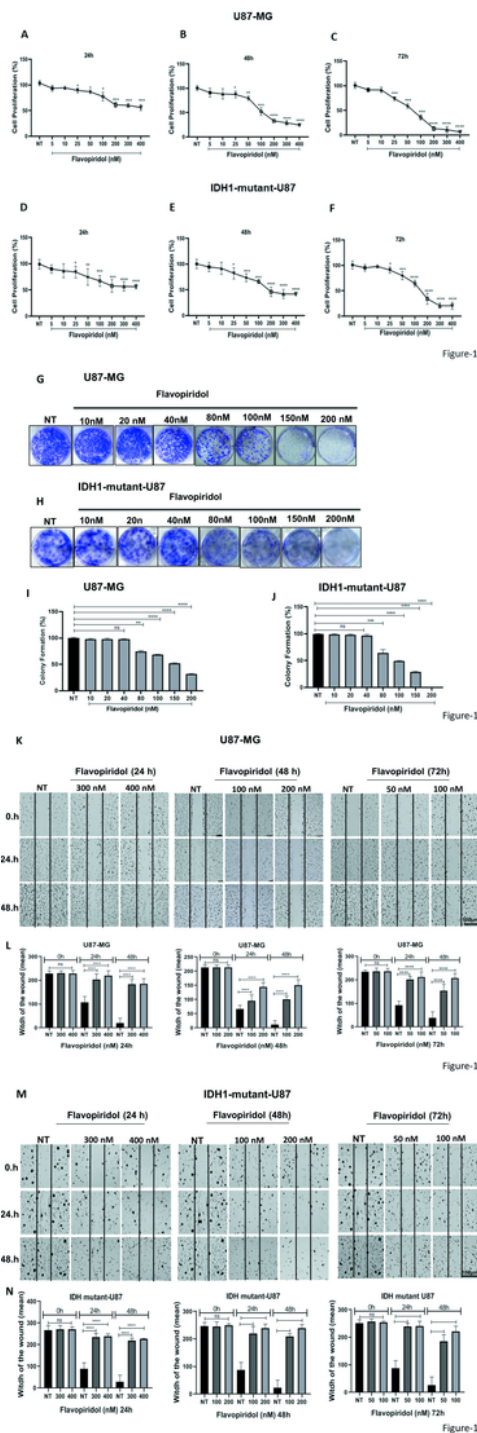


Figure 1

Flavopiridol treatment inhibits cell proliferation, colony formation and migration in GBM cells. Cells were treated with increasing concentration of flavopiridol, and cell proliferation was evaluated after 24 h, 48 h and 72 h by MTS assay (A-F) for colony formation in U-87MG (G, I) and IDH-mutant-U87 (H, J) cells. Cells were treated with increasing concentration of flavopiridol and evaluated after 24 h, 48 h and 72 h for

migration in U-87MG (K-L) and IDH-mutant-U87 (M-N) cells. All experiments were independently repeated at least twice. (ns: non-significant, *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

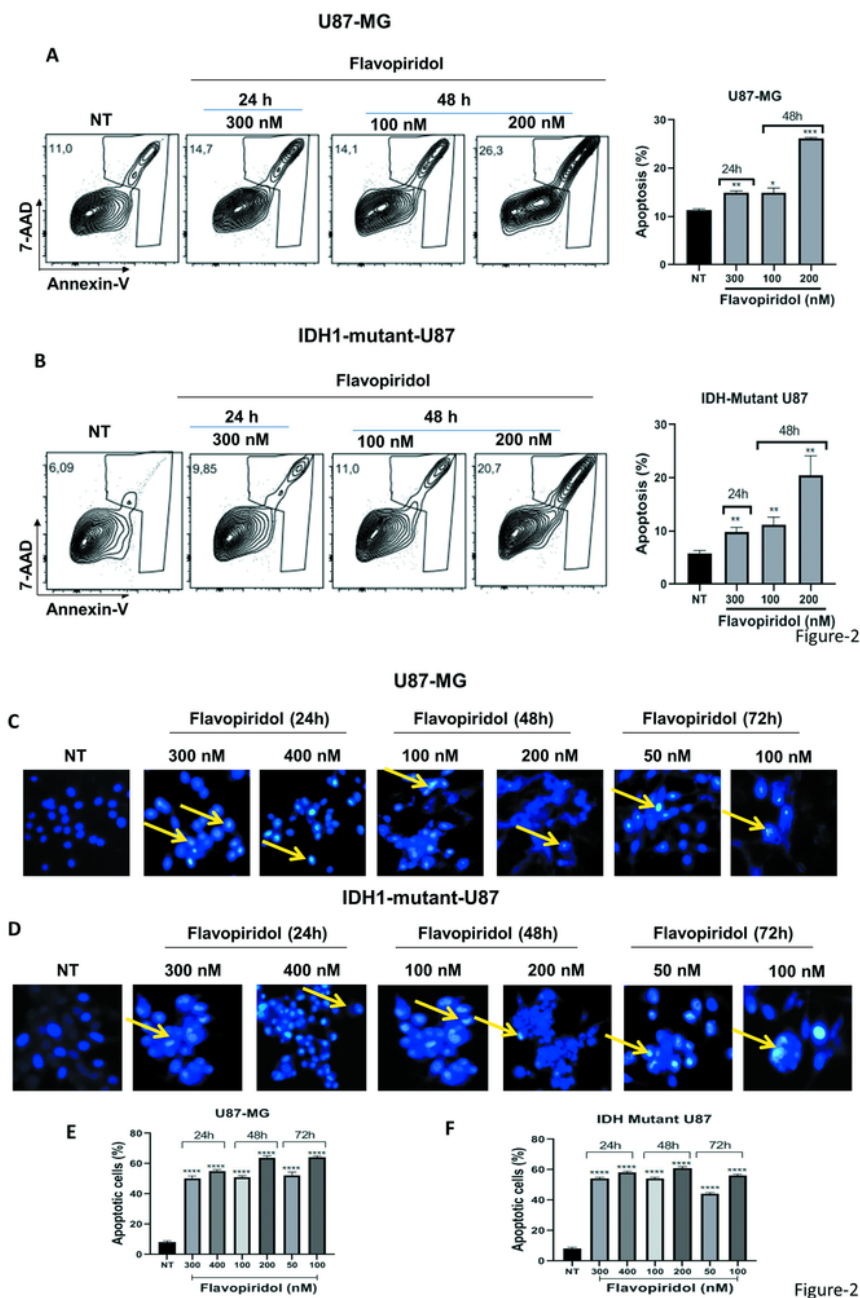


Figure 2

Flavopiridol leads to nuclear morphologic changes and induces apoptosis in GBM cells. Cells were treated with increasing concentration of flavopiridol and stained with AnnexinV and 7AADA after 24 h, 48 h.

Positively stained cells were quantified with BD FACSARIAIII. A representative flow plot was shown (A, B). Cells were treated with increasing concentration of flavopiridol and evaluated after 24 h, 48 h and 72 h for morphologic changes in cell nuclei using Hoechst staining under fluorescence microscopy (Original magnification, 20 ×) (C-F). Arrows indicate nuclear fragmentation. (*p <0.05, **p <0.01, ***p <0.001).

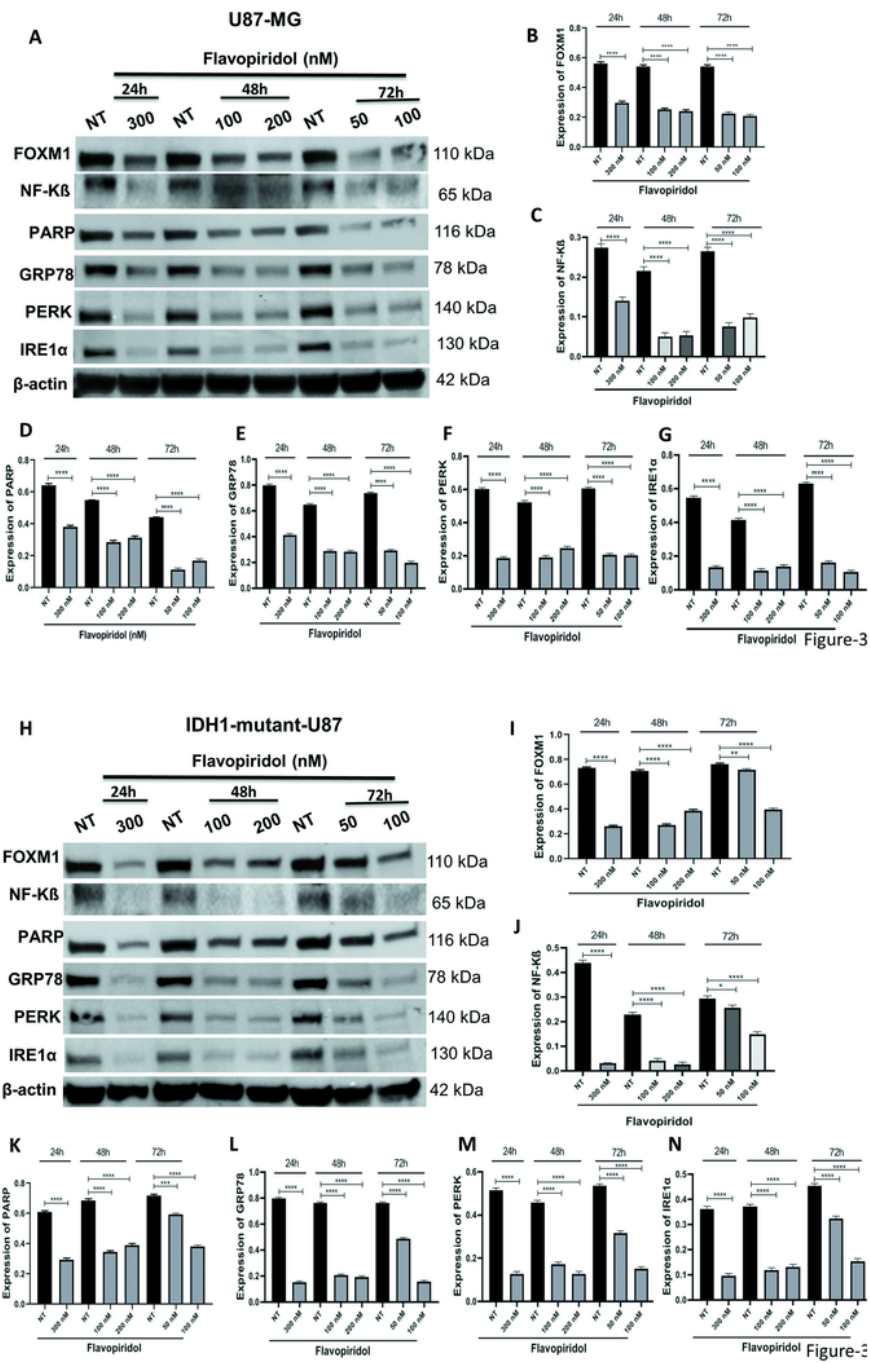


Figure 3

Falovopirdol inhibits expression of FOXM1, NF κ B, PARP and UPR mediators in GBM cells. Cells were treated with increasing concentration of flavopiridol. Expressions of FOXM1, NF κ B, PARP and UPR mediators (GRP78, PERK, IRE1 α) proteins were determined by Western blot in U87-MG (A) and IDH-mutant-U87 (H) cells. β 1-actin was used as a loading control. Protein expression intensities were evaluated by densitometric analysis in U87-MG (B-G) and IDH-mutant-U87 (H-N) cells. (**p <0.001, ****p <0.0001). All experiments were independently repeated at least twice.

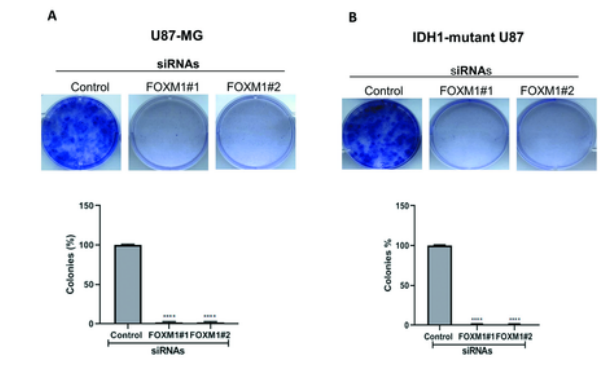


Figure-4

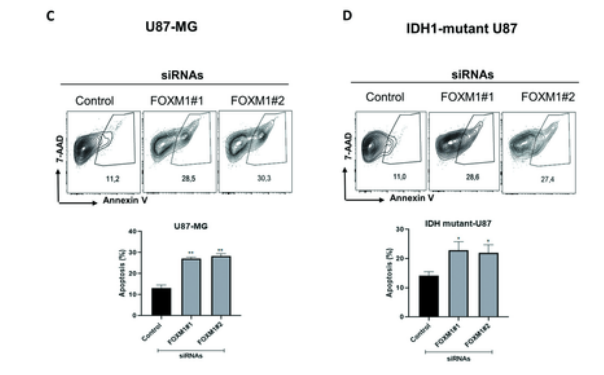


Figure-4

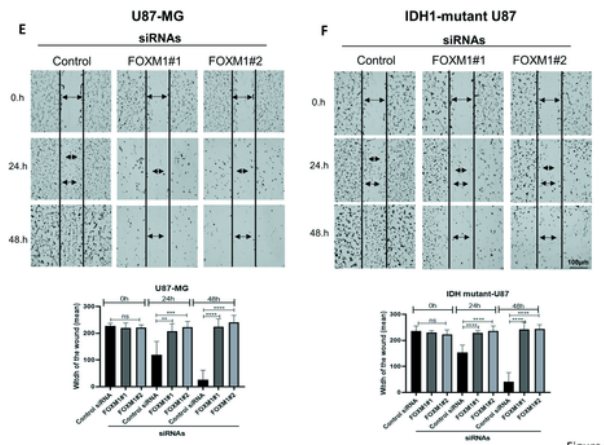


Figure-4

Figure 4

Knockdown of FOXM1 inhibits colony formation and migration and induced apoptosis in GBM cells. Cells were transfected with two different FOXM1-siRNAs (50 nM) targeting FOXM1 or control (non-targeting) siRNA. Knockdown of FOXM1 by siRNAs significantly inhibited colony formation in both U87-MG (A) and IDH-mutant-U87 (B) cells. The colony areas were measured densitometrically at the end of the 14 days in U87-MG (A) and IDH-mutant-U87 (B) cells. Cells were treated with control and FOXM1 siRNAs and stained with AnnexinV and 7AAD after 72 h. Positively stained cells were quantified with BD FACSARIAIII. A representative flow plot was shown (C, D). Cell migration was measured by a scratch wound healing assay. A single scratch was made in the center of the confluent cell monolayer and cells were transfected with indicated siRNAs. The cell migration was monitored for 24h -48h 48 h and visualized by light microscopy. Images were taken immediately (0 h), and after 24h and 48 h of scratching the cultures. The cell migration was monitored for 24h -48h and visualized by light microscopy. Images were taken immediately (0 h), and after 24h and 48 h of scratching the cultures. The data were expressed as average distance between the edges of the gap (E-F). (ns: non-significant, **p <0.001, ****p <0.0001).

(B-G) and IDH-mutant-U87 (H-N) cells. All experiments were independently repeated at least twice. (p <0.01, ***p <0.001, ****p <0.0001).

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