OPEN ACCESS Freely available online

Biochemistry & Pharmacology: Open Access

Research Article

Dual Inhibition of Mek5 and Mek1/2 or Pi3k Pathways Decreases Cell Viability, Proliferation, Migration, and Stemness, and Induces Mesenchymal to Epithelial Transition in Glioblastoma Multiforme

Akshita B. Bhatt[1], Katie Anna[1], Seraina Schottland[1], Jordan Martin[1], Thomas D. Wright[1], Mohit Gupta[2], Margarite D. Matossian[3], Deniz A. Ucar[4], Suravi Chakrabarty[2], Van Barnes[3], Saloni Patel[2], Lucio Miele[4], Matthew E. Burow[3], Patrick T. Flaherty[2], Jane E. Cavanaugh[1*]

[1]*Department of Pharmacology, Duquesne University, Pittsburgh*

[2]*Department of Medicinal Chemistry, Duquesne University, Pittsburgh*

[3]*Department of Medicine, Tulane University, USA*

[4]*Department of Genetics Louisiana State University Health Sciences, USA*

ABSTRACT

Glioblastoma multiforme (GBM) is an aggressive brain cancer with poor prognosis of less than 15 months. Mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K) pathways are important regulators of tumor progression, epithelial to mesenchymal transition (EMT), and metastases in GBM. Additionally, epigenetic alterations in histone deacetylase and bromodomain extra-terminal proteins are known to enhance EMT and proliferation in several cancers, including glioblastomas. Our results from bioinformatics analyses of healthy versus GBM tumor samples revealed that there was a significant enrichment in genes involved in EMT and proliferation in GBM versus healthy condition. Moreover, MAPK7, a downstream target of the MAPK pathway, which codes for extracellular signal-regulated kinase (ERK)5 was significantly upregulated in GBM tissues compared to healthy control.

The goal of the current research is to develop therapeutic interventions to reverse EMT and proliferation in GBM. Since crosstalk between ERK5 and AKT has been previously noted, we evaluated the effect of single and dual ERK5 and AKT inhibition using known pharmacological inhibitors XMD8-92 and ipatasertib on U87MG cell viability. The effect of novel dual MEK5/PI3K inhibitor on cell proliferation was evaluated in combination with bromodomain 4 (BRD4) inhibitor CPI-203. Moreover, we examined the effect of previously published novel MEK1/2 and/or MEK5 inhibitors on mesenchymal to epithelial transition (MET), the reverse of EMT, in PTEN-mutant U87MG glioblastoma cells, which have a mesenchymal phenotype.

Keywords: Glioblastoma multiforme, ERK5, AKT, Targeted therapy, EMT

INTRODUCTION

Glioblastoma multiforme (GBM) is a very aggressive form of cancer with median survival of only 14-15 months despite standard therapy consisting of surgery, adjuvant radiation, and chemotherapy. Infiltration into surrounding tissue decreases the success of surgical removal of the tumor and further increases the rate of recurrence [1,2]. Hyperactivation of PI3K and MAPK pathways is a frequent event in most GBM cases. Moreover, the mesenchymal subtype of GBM is driven by activation of the ERK1/2 pathway [3]. The Cancer Genome Atlas identified 4 subtypes of GBM: The classical subtype, characterized by EGFR overexpression, the proneural GBM with isocitrate dehydrogenase (IDH) and p53 mutations, the mesenchymal subgroup with high neurofibromatosis type 1 (NF-1) expression, and a neural subtype with no specific gene mutations [4]. Irrespective of the subtype,

Correspondence to: Jane E. Cavanaugh, Department of Pharmacology and Toxicology, Duquesne University, Pittsburgh, Tel: 1-412-760-3503; E-mail: cavanaughj@duq.edu

Received date: June 08, 2021; **Accepted date:** June 22, 2021; **Published date:** June 29, 2021

Citation: Bhatt AB, Anna K, Schottland S, Martin J, Wright TD, Gupta M, et al. (2021) Dual Inhibition of Mek5 and Mek1/2 or Pi3k Pathways Decreases Cell Viability, Proliferation, Migration, and Stemness, and Induces Mesenchymal to Epithelial Transition in Glioblastoma Multiforme. Biochem Pharmacol. 8:218.

hyperactivation of PI3K and MAPK pathways is a frequent event in most GBM cases. Moreover, the mesenchymal subtype of GBM is driven by activation of the ERK1/2 pathway [3].

The goals of the current research are to identify therapeutic targets for the treatment of glioblastoma multiforme, which has the worst prognosis and decreased overall survival compared to other brain cancers. There are no current therapies that are effective to treat patients with GBM. There is an urgent need to identify relevant targets that can be inhibited therapeutically to improve prognosis of GBM patients. MAPK and PI3K pathways are recently identified to promote GBM progression [5]. We first utilized a bioinformatics approach to compare genes downstream of MAPK and PI3K pathways and those involved EMT and proliferation in healthy versus GBM tumor samples using publicly available datasets. The MAPK7 gene codes for the newest member of the MAPK pathway, extracellular signal-regulated kinase ERK5, and is significantly up regulated in GBM tumor samples compared to healthy control. GBM tumor samples were enriched in EMT markers and genes involved in cell proliferation, which are known downstream targets of MAPK and PI3K pathways.

Activation of AKT is an important event in GBM progression due to mutation in PTEN or PI3K. One of the limitations of PI3K pathway inhibitors is compensatory increases in alternative pathways, including the MEK5-ERK5 pathway [6]. This necessitates the development of novel and relevant combination strategies to target GBM. We have previously shown that dual inhibition of AKT and ERK5 or triple inhibition of AKT, ERK5, and BRD4 is a relevant strategy to target triple-negative breast cancer (TNBC) [6]. Moreover, the crosstalk between ERK5 and AKT pathways has been previously noted in neuroblastomas [7]. Therefore, we evaluated the effect of dual inhibition of ERK5 and AKT pathways on cell viability, proliferation, and migration in GBM. MYC is a major oncogenic driver in GBM, which was found to be upregulated and positively correlated with MAPK7 in GBM patient samples. Bromodomain inhibitors were developed with a rationale to target the MYC oncogene in cancer [8]. Therefore, a dual MEK5/PI3K inhibitor J19 (MG-3-81) [9] was used alone and in combination with CPI-203, a BRD4 inhibitor. We also examined the effects of previously reported novel inhibitors of MEK5 or MEK1/2 pathways, which reversed the mesenchymal phenotype of TNBC [10] on EMT in GBM.

MATERIALS AND METHODS

Cell culture

U87MG cells were cultured in MEM media supplemented with 5% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA). The cells were maintained at 37°C and 5% CO2 as per standard manufacturer's protocol.

Inhibitor treatment and EGF stimulation

Cells were cultured in a 6-well plate (250,000 cells/well) for 24 hrs. To examine kinase activity or inhibition, the cells were serum-starved for 18-24 h. The inhibitors XMD8-92 (Tocris, Minneapolis, MN) and trametinib (Selleckchem, Houston, TX) were added for 30 minutes prior to EGF (100 ng/ml) stimulation for 15 minutes as previously described [11]. Cells were lysed and examined for kinase activation using standard western blot procedures. The cells were lysed in ice-cold 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA) buffer and 0.1 M PMSF. The lysates were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was stored at -80°C until further analyses. The lysates were denatured using β-mercaptoethanol. Bradford (Bio-Rad, Hercules, CA) protein assay was performed to determine the protein concentrations in the lysates. 30 µg of protein was loaded on 8% SDS-PAGE gels. The gels were transferred to nitrocellulose membranes. The membranes were incubated in casein blocking buffer at room temperature for 1 h. Primary antibodies ERK5, ERK1/2, pERK1/2 (phospho-p44/42), pAKT, AKT, p21, CD133, and β-tubulin (Cell Signaling Technology) were added and the membranes were incubated at 4°C overnight. The membranes were washed in PBS-0.1% tween solution three times at 10-minute intervals. Secondary antibodies were added, and the membranes were incubated for 1 h and washed three times at 10-minute intervals at room temperature. The membranes were washed with PBS and scanned using an Odyssey (LI-CR, Lincoln, NE) imager at 700 and 800 nm wavelength. The blots were quantified using Image Studio Lite (LI-COR Biosciences).

Immunofluorescence assay

U87MG cells were cultured in 96-well plates (5,000 cells/well). After 24 h of plating, treatments were added for 72 h. The media was removed; cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were washed and incubated with blocking buffer for 1 hour at room temperature. β-tubulin and Ki67 primary antibodies were added at f 1:750 dilution and the plate was incubated at 4°C overnight. The cells were washed with 1X PBS thrice at 5-minute intervals. Goat anti-mouse Alexa Flour 488 nm and goat anti-Rabbit Alexa Flour 555 nm (1:1000, Invitrogen) secondary antibodies, counterstained with Hoechst (Fisher) were added for 1 hour at room temperature. Cells were washed with PBS three times at 5-minute intervals. The pictures were taken using the EVOS microscope (Thermo Fisher Scientific, Waltham, MA) at 10X magnification.

MTT cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5v -diphenyltetrazolium bromide) assay was performed to determine cell viability. Cells were seeded at a density of 5,000 per well in 96-well plates containing 90 µl of full media for 24 h and then treated with increasing concentrations of ipatasertib, trametinib, or XMD8-92 for 72 h. 10 µL of MTT (Acros, Cat. No. 298-93-1) solution (5 mg/ml in phosphate-buffered saline, PBS) was added to each well and the plate was incubated at 37°C for 3 h. MTT solution was removed from each well. 100 µl of DMSO was added to each well for 10 min under agitation to dissolve the formazan crystals. Absorbance was measured at a wavelength of 570 nm. Synergy calculations were performed using CompuSyn software.

Crystal violet staining and spindle index calculation

Cells were seeded at a density of 5,000 cells/well in a 96-well plate. After 24 hours of plating, cells were treated with compounds and allowed to grow for 3 days after treatment. Media was aspirated and cells were washed with PBS, fixed with 4% paraformaldehyde (50 µl per well) for 15 min. The cells were washed once with PBS and stained with crystal violet (50 µl per well) for 15 min. The cells were washed with 50 µl PBS three times. Pictures were captured using EVOSTM FL inverted microscope (Life Technologies). Spindle Index Calculation Spindle indices (SI) of individual cells were calculated from at least 200 cells per treatment from a minimum of three images as the ratio of length (l) to width (w); SI=l/w of each cell. Length and width were measured using the Image J software, U. S. National Institutes of Health, Bethesda, Maryland, USA. Cells with SI<3 were considered as epithelial. The percentage of cells <3 was calculated as the ratio of the number of cells with spindle index <3 to the total number of cells per image as described previously [10,12].

Statistical analyses

Genomics data were analyzed using R2: Genomics analysis and visualization platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). One-way or two-way analysis of variance (ANOVA) with Bonferroni post-hoc correction was applied to determine statistical significance across different concentrations of individual drugs compared to the control (DMSO or GFP) or to the individual drug where combination treatment was performed. GraphPad Prism version 7.03 for Windows (GraphPad Software La Jolla, California) was used for statistical analyses.

RESULTS

MAPK7 gene expression is significantly up regulated in GBM tumor samples compared to healthy groups

Glioblastoma multiform (GBM) was identified to have the worst prognosis among other forms of cancers that originate in the brain (Figure 1A). MAPK7 gene, which codes for protein ERK5 was found to be significantly up regulated in GBM tumor samples compared to healthy group (Figure 1B). In contrast, MAPK1 and MAPK3 genes were significantly down regulated in GBM vs. healthy group. Epithelial cell markers CDH1 and keratin 1 (KRT1) were significantly down regulated, whereas tight junction protein (TJP1) gene expression did not decrease in GBM versus healthy group. Mesenchymal cell marker vimentin (VIM) and stem cell marker PROM1 were significantly up regulated. As expected, genes involved in cell proliferation KI67, CCND1, and PCNA were significantly up regulated in GBM versus healthy group. Upstream regulator of MAPK pathway, EGFR was significantly up regulated while there was no increase in PIK3CA gene; however, there was a significant decrease in PTEN gene, which inhibits AKT activation by dephosphorylating. There was a significant increase in AKT1 and AKT2 and decrease in AKT3 gene expression (Figure 1B). Moreover, MAPK and pathways involved in regulation of actin cytoskeleton were significantly up regulated in GBM compared to healthy tissues (Supplementary Table 1).

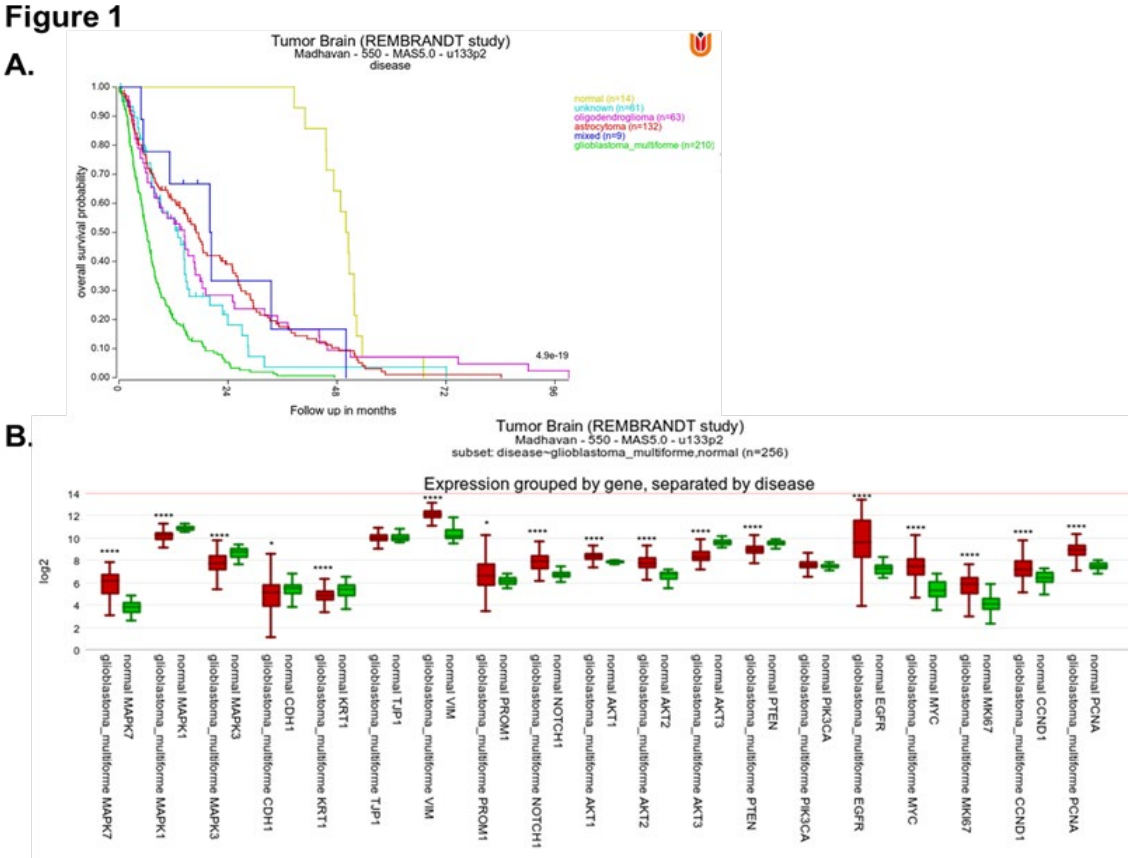


Figure 1: Comparison of genes downstream of MAPK and PI3K pathways in tumors derived from GBM patients versus healthy groups (A) Kaplan-Meier survival analysis in different subtypes of brain cancer (B) Gene expression analyses in healthy tissues versus GBM patient samples.

MAPK7 gene expression positively correlates with the EMT markers in GBM tumors

ERK5 is known to antagonize the decrease in MYC induced by KRAS suppression, indicating that ERK5 may be upstream of MYC [13]. Interestingly; there was a significant positive correlation between MAPK7 and MYC in GBM. To understand whether ERK5 regulates genes involved in EMT and stemness, MAPK7 gene expression was correlated to stem cell marker PROM1 (gene that codes for CD133) and epithelial cell marker CDH1 (gene that codes for E-cadherin). MAPK7 gene expression negatively correlated with CDH1 and positively correlated with PROM1, NOTCH1 and mesenchymal markers VIM and ZEB1 (Figure 2), suggesting a role for ERK5 in mediating EMT and stemness in GBM.

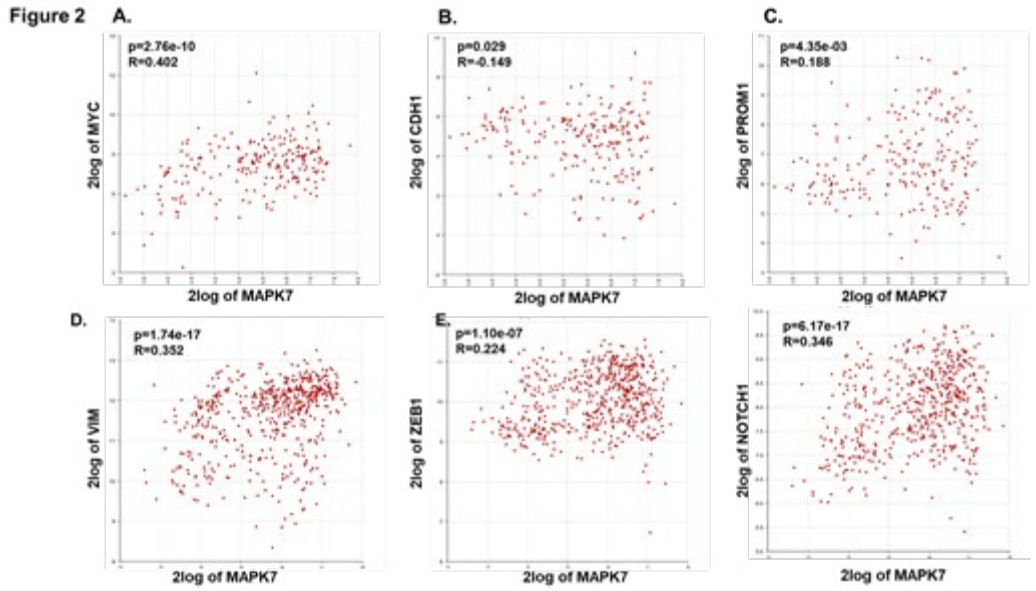


Figure 2: MAPK gene correlation with EMT and stemness markers. Gene correlation between MAPK7 and (A) PROM1 (B) CDH1, (C) MYC, (D) VIM (E) ZEB1, or (F) NOTCH1.

MAPK1, MAPK7, AKT1, and AKT2 gene expression is associated with poor patient survival in GBM

Kaplan Meier survival analysis was performed to examine the association of MAPK and PI3K pathways on overall survival in GBM using publicly available datasets. High MAPK1 (gene that codes for ERK2), MAPK7 (gene that codes for ERK5), AKT1 and AKT2 expression was associated with worse patient outcome whereas high MAPK3 (gene that codes for ERK1) and AKT3 expression was associated with better patient outcome (Figure 3). Overall, these data suggest that ERK5 and AKT1 and 2 are important targets in GBM.

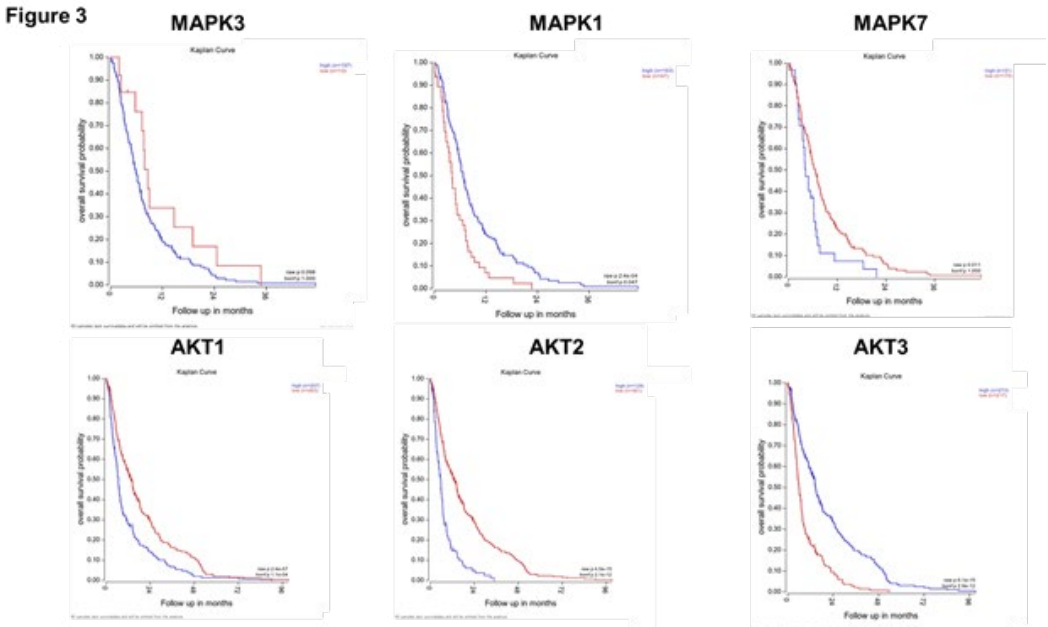


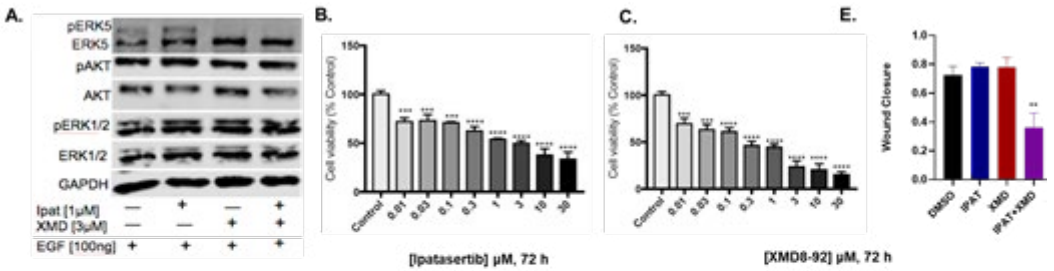
Figure 3: MAPK3, MAPK1, and MAPK7 expression correlates with poor patient survival in GBM.

Dual inhibition of ERK5 and AKT pathways synergistically reduces cell viability in GBM

U87MG cells are PTEN mutant and may rely on the AKT pathway for survival and proliferation. Since crosstalk between AKT and ERK5 may exist, the effects of ipatasertib, an AKT inhibitor and XMD8-92, an ERK5 inhibitor alone and in combination were examined on kinase activation, cell viability, and migration. To determine the specificity of kinase inhibitors, U87MG cells were serum starved for 18 hours after 24 hours of cell seeding and treated with kinase inhibitors for 30 minutes. Cells were stimulated with EGF for 15 minutes. Cell lysates were collected and examined for ERK1/2, ERK5, and AKT activation. As expected, XMD8-92 and XMD8-92+ipatasertib groups decreased ERK5 activation with no effect on AKT or ERK1/2 activation. Ipatasertib targets the ATP-site and keeps AKT in its active form and decreases downstream signaling by AKT. This may explain while there was no decrease in AKT activation, there was still a decrease in cell viability after treatment with ipatasertib. Ipatasertib modestly increased ERK5 activation (Figure 4A). Neither of the inhibitors altered ERK1/2 activity. Targeting AKT alone led to compensatory increase in ERK5 activation, which was inhibited in the Ipat+XMD8-92 group compared to control (Figure 4A).

While both ipatasertib and XMD8-92 were effective at decreasing cell viability in a concentration-dependent manner (Figures 4B and 4C), combination of ipatasertib with XMD8-92 produced a greater reduction in cell viability. 1:3 combination of ipatasertib and XMD8-92 was more effective than 1:1 ratio at increasing the fraction affected as calculated by using CompuSyn software (Figure 4D). While ipat or XMD8-92 did not decrease cell migration alone, the combination significantly decreased cell migration in U87MG cells. Ipat+XMD combination did not significantly decrease the proliferative fraction (data not shown), indicating that alternative pathways may regulate cell proliferation in GBM.

Figure 4



D.

Cell line	Combination (Ipat+XMD)	Fraction affected (Fa)	Combination index (CI)	Synergy designation
U87	0.1, 0.1	0.614	0.134	Strong synergy
	0.3, 0.3	0.571	0.699	Strong synergy
	1, 1	0.543	3.311	Antagonism
	3, 3	0.79	0.285	Strong synergy
	0.1, 0.3	0.571	0.667	Synergy
	1, 3	**0.815**	**0.171**	**Strong synergy**

Figure 4: Dual ERK5 and AKT decreases cell viability and migration in U87MG GBM cells. (A) Western blot analysis of ERK5, ERK1/2, and AKT activation by EGF in U87MG cells (B) Cells were treated with XMD8-92 and ipatasertib at increasing concentrations for 72 hours. Data represent the ± SEM of three different experiments for each inhibitor compared to DMSO control. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs DMSO control group determined by one-way ANOVA with the Bonferroni post hoc test. (C) Combination index table for synergy determination. (D) Effect of ipatasertib and XMD8-92 at 1 and 3 μM concentration, respectively on cell migration in U87MG cells. Scratches were made after 24 hours of cell seeding (0h) and cells were treated with the kinase inhibitors for 24 hours. Cells were imaged at the time of scratch (0 h) and after 24 hours from the time of scratch (20X magnification).

Dual PI3K/MEK5 inhibitor+CPI203 combination significantly decrease cell proliferation via p21 restoration in GBM

BRD4 regulates cell cycle progression via upregulation of c-Myc and cyclinD1, which are also downstream targets of MAPK pathways. Several dual PI3K/MEK5 thiophene analogs were developed as novel therapeutic interventions to target cancer. In this study, we examined the effect of novel dual MEK5/PI3K inhibitor J19 (MG-3-81) in combination with BRD4 inhibitor CPI-203 on cell proliferation. Ipatasertib, J19 (MG-3-81), or CPI-203 alone did not significantly decrease the proliferative fraction as determined by taking the ratio of Ki67+ cells to Hoechst+ cells. While ipatasertib did not potentiate the effect of CPI203 on cell proliferation, J19 (MG-3-81)+CPI-203 combination significantly decreased proliferative fraction to a greater extent compared to either drug alone (Figure 5A). The decrease in cell proliferation correlated with an increase in p21, a cell cycle inhibitor (Figure 5B).

Figure 5

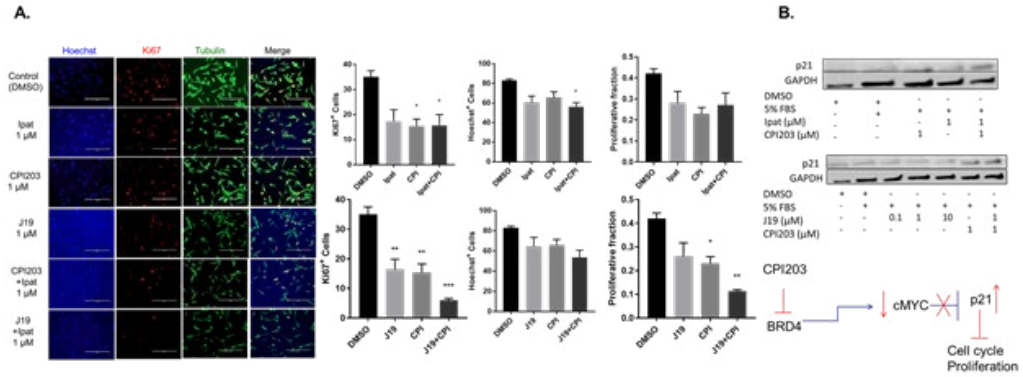


Figure 5: Effect of ipatasertib and J19 (MG-3-81) alone and in combination with CPI-203 on cell proliferation and p21 expression in U87MG cells. (A) Cells were treated with indicated inhibitors for 72 hours (20X magnification). Proliferative fraction was evaluated as the number of Ki67 positive cells divided by the number of Hoechst positive cells. Data represent the ± SEM of three different experiments. *p<0.05; **p<0.01; ***p<0.001 vs. DMSO control group determined by one-way ANOVA with the Bonferroni post hoc test.

Dual MEK1/2 and MEK5 inhibitor (s) and LBH589 (HDAC inhibitor) induce MET in U87MG glioblastoma cells

U87MG cells were treated with novel MEK1/2 inhibitor (SC-1-122), MEK5 inhibitor (SC-1-181), dual MEK1/2 and MEK5 inhibitor (SC-1-151), LBH589, trametinib, or XMD8-92 for 72 hours. The phenotypic switch from mesenchymal to epithelial was most prominent in SC-1-151, trametinib, and LBH-589-treated groups (Figures 5A and 5B). We hypothesized that targeting the AKT pathway may be a relevant strategy to reverse EMT; however, ipatasertib, J19 (MG3-81), or CPI-203 did not reverse EMT in these cells (Figure 6). Therefore, the ERK1/2 and ERK5 pathways may play a larger role in the EMT than the AKT pathway.

Figure 6 **Dual MEK1/2 and MEK5 inhibition induces MET in U87MG cells**

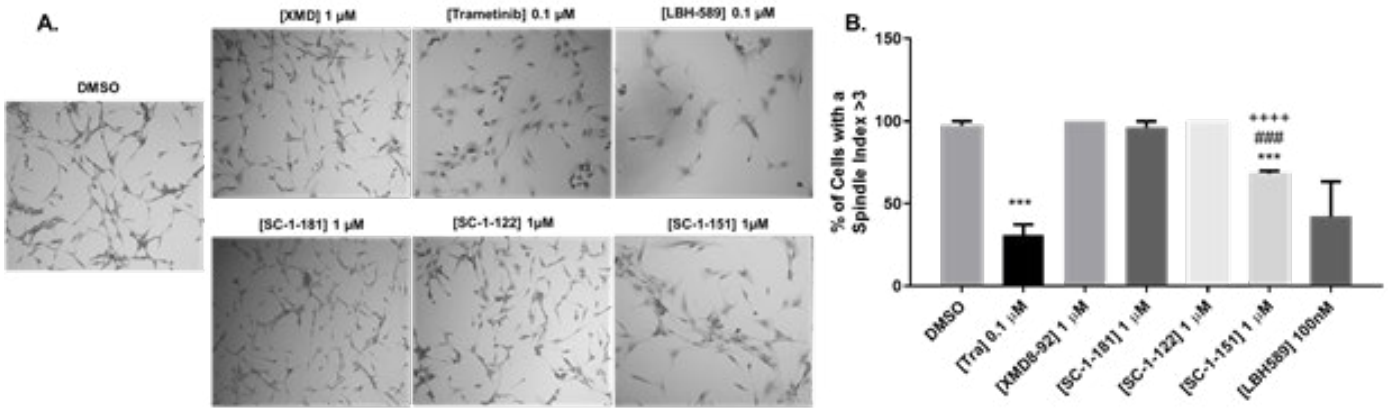


Figure 6: SC-1-151 induces MET in U87MG GBM cells. (A) Morphology images captured after 72 hours of drug treatment. Cells were stained with crystal violet. (B) Spindle index was calculated in a blinded manner and % cells with spindle index > 3 were plotted for individual treatment. Data indicate ± SEM of experiments run in triplicate ***p<0.001; vs DMSO control group, ###p<0.001 vs SC-1-181; ++++p<0.0001 vs SC-1-122 determined by one-way ANOVA with the Bonferroni post hoc test

Trametinib decreases ERK5 activation, cell viability, migration, and colony formation in GBM

The MEK1/2 inhibitor trametinib was most effective at reversing the mesenchymal phenotype of U87MG cells to epithelial. Therefore, we examined the effect of this inhibitor on viabililty, proliferation, and migration in glioblastoma cells. U87MG cells were serum starved for 18 hrs and treated with trametinib at 0.1 and 1 μM concentrations for 30 minutes. Cells were then stimulated with EGF for 15 minutes. Lysates were collected and examined for ERK5, ERK1/2, and AKT activation. As expected, trametinib decreased ERK1/2 activation by EGF. Surprisingly, trametinib also inhibited ERK5 activation by EGF (Figure 7A).

MTT, migration, and colony formation assays were performed to examine the effects of trametinib on U87 cells in 2D and 3D cultures. Trametinib modestly decreased cell viability at 10 μM concentration (Figure 7B) and significantly inhibited cell migration, CD133 expression, and colony formation, consistent with its effect on MET in U87MG cells (Figures 7C and 7D).

Figure 7

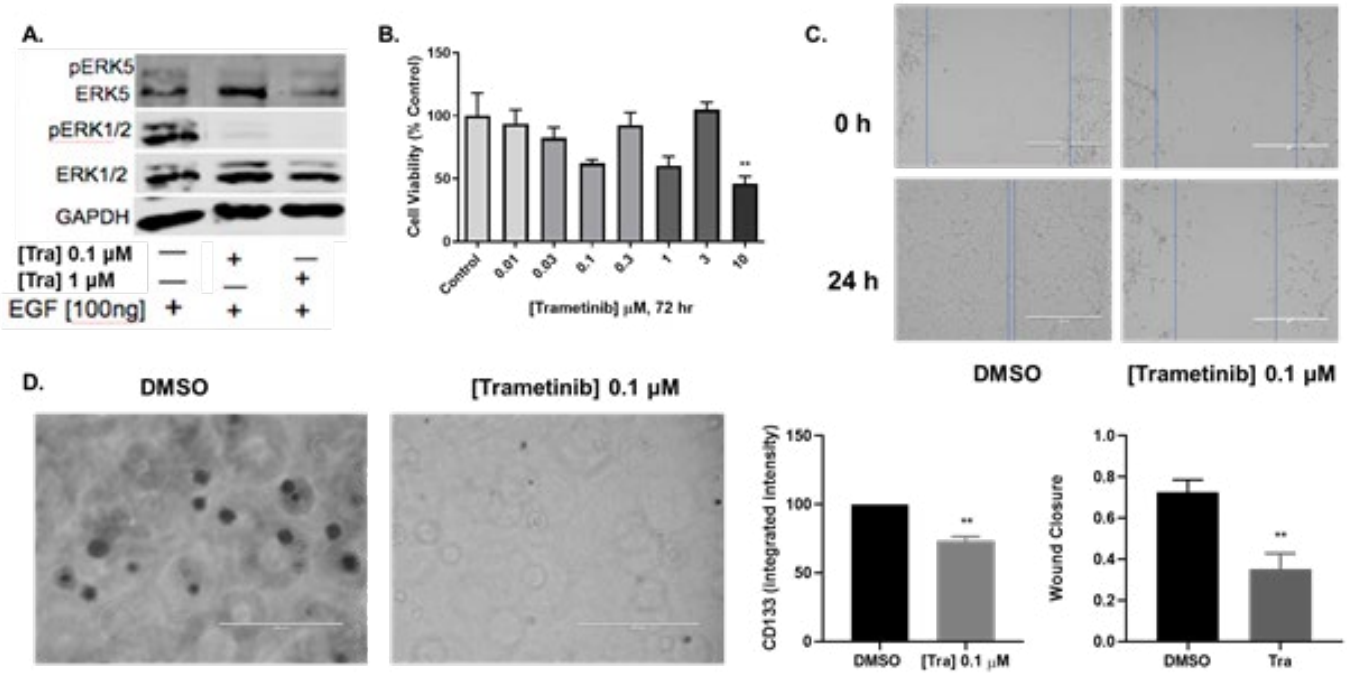


Figure 7: Trametinib decreases (A) ERK5 and ERK1/2 activation (B) cell viability (C) migration (D) colony formation and CD133 expression in U87MG GBM cells. **p<0.01 vs control group determined by Student's t-test.

MEK5 inhibitor SC-1-181 decreases CD133 and colony formation in GBM

ERK5 is known to regulate the extracellular matrix and stemness in some cancers. Therefore, we examined the effect of the MEK5 inhibitor on CD133, an essential regulator of stemness in GBM [14] and colony formation. SC-1-181 significantly decreased CD133 in a dose-dependent manner (Figure 8A). The decrease in CD133 correlated with a decrease in colony formation (Figure 8B). J19 (MG-3-81) also decreased colony formation in GBM.

Figure 8

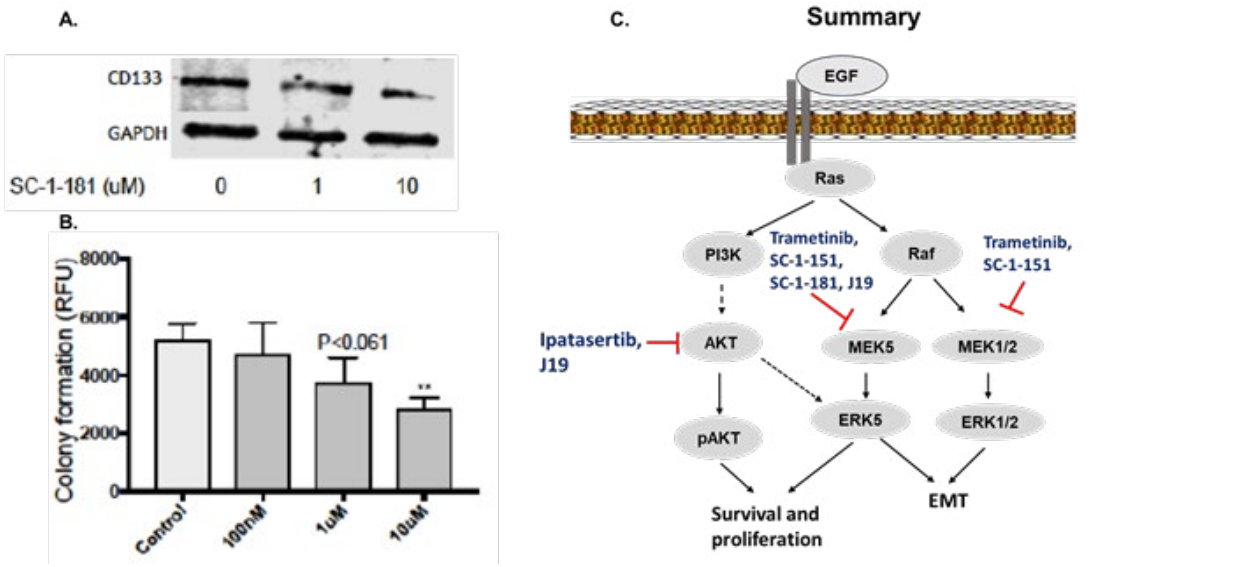


Figure 8: SC-1-181 decreases (A) CD133 expression (B) colony formation in U87MG cells. **p<0.01 vs DMSO control group determined by one-way ANOVA with the Bonferroni post hoc test. (C) Putative signaling for regulation of cell viability, EMT, and stemness via the MAPK and PI3K pathways in GBM.

DISCUSSION

Glioblastoma multiforme is an aggressive disease with limited therapeutic options. ERK1/2, ERK5, and AKT pathways are important targets in glioblastoma. However, the crosstalk between ERK5 and AKT and effects of MAPK inhibitors on proliferation, EMT, and stemness in glioblastoma are not well-understood. Therefore, we examined the effect of novel and known MAPK pathway inhibitors in combination with PI3K pathway inhibitor (s) on viability, migration, EMT, and stemness in U87 GBM cells.

Recent studies suggest that ERK5 expression and activation are crucial events in regulating EMT in several cancers [15,16] and targeting the MAPK pathways has been shown to reverse EMT in breast cancer [10,17]. One goal of this research was to identify pathways that were significantly upregulated in GBM compared to healthy controls, so as to therapeutically target the aberrant pathways. Recent evidence suggests that EGFR, PI3K, and PTEN mutations4 are common oncogenic drivers in GBM, making GBM a relevant disease model to study ERK1/2, ERK5, and AKT protein kinases as relevant drug targets in GBM. From our research, using publicly available datasets from [18]. We examined specific components of MAPK and PI3K pathways, which were upregulated in GBM versus healthy control.

MAPK7 was found to be significantly upregulated in GBM versus healthy control. Therefore, we correlated MAPK7 gene expression to EMT and proliferation markers. There was a significant increase in the upstream regulator of MAPK pathways, epidermal growth factor receptor (EGFR), in agreement with previous reports [19]. PTEN deletion is a common event in GBM.4 Since PTEN expression was significantly downregulated in GBM versus healthy control, AKT may be a relevant target in GBM. Moreover, high AKT1 and AKT2 expression was associated with worse patient outcome. Since high MAPK3 and AKT3 gene expression correlated with better patient outcome, it may be important to develop isoform-specific pharmacological inhibitors of ERK1, AKT1, or AKT2, which spare ERK2 or AKT3 activity. Isoform-specificity of inhibitors is currently being investigated in our laboratory and others [20]. Data suggest it may be important to determine which tumor subtypes may benefit most from isoform-specific compounds.

While we obtained information about putative drug targets by utilizing a bioinformatics approach, these observations do not provide insights into whether there is a subsequent increase in protein expression or activation in different components of the MAPK and PI3K pathways. Therefore, we utilized an in vitro approach to inhibit these pathways using known and novel inhibitors of the MAPK and PI3K pathways to examine their effect on kinase activation, proliferation, EMT, and stemness. To understand whether targeting ERK5, ERK1/2, and/or PI3K pathways was promising in GBM, we utilized PTEN-mutant U87MG cells with moderate EGFR expression and a mesenchymal phenotype [21,22]. In addition to genes involved in the regulation of proteins that promote EMT, cell proliferation genes downstream of MAPK, AKT, and bromodomain pathways were significantly enriched in GBM tumors compared to healthy control. Compensatory activation of the AKT or BET proteins may reactivate MAPK-mediated proliferation in cancer cells [23]. Therefore, combination strategies were designed to target both MAPK and bromodomain proteins in U87MG cells. Similar to our findings in breast cancer, dual ERK5 and AKT inhibition significantly decreased cell viability and migration in U87MG cells. Novel dual PI3K/MEK5 inhibitor J19 (MG-3-81) was more effective in combination with BRD4 inhibitor CPI-203 to decrease cell proliferation compared to AKT inhibitor ipatasertib. Our data suggest that this decrease in cell proliferation was mediated at least in part via p21 restoration.

U87MG cells were especially susceptible to dual targeting of ERK1/2 and ERK5 pathways since treatment with dual MEK1/2 and MEK5 inhibitor significantly decreased the percentage of cells with SI>3 when compared with DMSO control or inhibition ofMEK1/2 or MEK5 alone (SC-1-122 or SC-1-181, respectively). Although MAPK7 gene expression significantly correlated with mesenchymal markers ZEB1, vimentin, and inversely correlated with epithelial marker E-cadherin, MEK5 inhibitor SC-1-181 did not reverse EMT in U87MG cells. This may be because ERK1/2 pathway is still active. Trametinib, a MEK1/2 inhibitor reversed EMT in U87MG cells. To our knowledge, this is the first study to show the effect of MEK1/2 inhibition on reversal of EMT in glioblastoma. A previous study has reported that downregulation of ERK5 by microRNA-200 suppressed EMT in GBM [24]. This may indicate that total ERK5 expression, rather than its activation may be a driver of EMT in GBM. The possible reasons why ERK5 inhibition alone did not reverse EMT may be because total ERK5 expression may regulate transcription of genes in the nucleus even in the absence of phosphorylated ERK5 in the cytosol [25]. Trametinib decreased total ERK5 expression even at short time points (Figure 7A), which may indicate that inhibition and decrease in total ERK5 expression may be necessary for full effect of trametinib on MET in U87MG cells. LBH589, a histone deacetylase inhibitor was used as a positive control since it known to reverse EMT in some cancers [26].

MEK1/2 inhibitor trametinib decreased ERK1/2 and ERK5 activation in response to EGF. ERK1/2 and ERK5 pathways share ~50% sequence homology at the N-terminal domain [27]. Trametinib-mediated decrease in ERK5 activation may be mediated via MEK1/2 inhibition. Trametinib decreased viability, migration, and colony formation in U87MG cells, indicating that dual MEK1/2 and MEK5 inhibition is a relevant strategy to target EMT in GBM. Overall, our data suggest that ERK1/2 and ERK5 are relevant targets to reverse the EMT and ERK5 and AKT are relevant targets to decrease cell viability and proliferation in glioblastoma cells with a mesenchymal phenotype. Cellular signaling pathway is outlined in Figure 8C.

ERK5 is known to regulate the extracellular matrix and stemness in cancer [15]. Increased stemness is also responsible

for chemotherapeutic drug resistance in GBM. Therefore, we evaluated the effect of the MEK5-ERK5 pathway inhibitor SC-1-181 on the stem cell marker CD133 and colony formation. SC-1-181 was effective in decreasing stemness and colony formation, indicating a crucial role of ERK5 in regulating stemness in GBM.

CONCLUSION

This is the first study to examine the effects of pharmacological inhibition of MEK5 and MEK1/2 or PI3K pathways on cell proliferation, migration, stemness, and EMT in GBM.

REFERENCES

1. Tran B, Rosenthal MA. Survival comparison between glioblastoma multiforme and other incurable cancers. J Neurosurg Society of Australasia. 2010;17 (4): 417-21.
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England J Med 2005;352 (10): 987-96.
3. Hannen R, Hauswald M, Bartsch JW. A rationale for targeting extracellular regulated kinases ERK1 and ERK2 in Glioblastoma. J Neuropath & Experiment Neurol. 2017;76 (10): 838-847.
4. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H. The somatic genomic landscape of glioblastoma. Cell. 2013;155 (2): 462-77.
5. Finch A, Solomou G, Wykes V, Pohl U, Bardella C. Advances in Research of Adult Gliomas. Int J Mol Sci. 2021; 22 (2): 924.
6. Wright TD, Raybuck C, Bhatt A, Monlish D, Chakrabarty S. Pharmacological inhibition of the MEK5/ERK5 and PI3K/Akt signaling pathways synergistically reduces viability in triple-negative breast cancer. J Cellular Biochem 2020;121 (2):1156-1168.
7. Umapathy G, El Wakil A, Witek B, Chesler L, Danielson L. The kinase ALK stimulates the kinase ERK5 to promote the expression of the oncogene MYCN in neuroblastoma. Science signaling 2014;7 (349): ra102.
8. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell. 2011;146(6): 904-17.
9. Gupta M, Shah D, Flaherty P, Wright T, Bhatt A. In Selective allosteric inhibition of MEK5: Novel target for cancer therapeutics, Abstracts of Papers of the American Chemical Society, Amer Chemical Soc 1155 16th St, Nw, Washington, DC 20036 USA: 2018.
10. Bhatt AB, Gupta M, Hoang VT, Chakrabarty S, Wright TD. Novel diphenylamine analogs induce mesenchymal to epithelial transition in triple negative breast cancer. Frontiers in Oncology. 2019;9 (672).
11. Chakrabarty S, Monlish DA, Gupta M, Wright TD, Hoang VT. Structure activity relationships of anthranilic acid-based compounds on cellular and in vivo mitogen activated protein kinase-5 signaling pathways. Bioorganic & medicinal chemistry letters. 2018; 28(13):2294-2301.
12. Koo V, El Mekabaty A, Hamilton P, Maxwell P, Sharaf O. Novel in vitro assays for the characterization of EMT in tumourigenesis. Cellular oncology. J Int Soc Cellular Oncol. 2010;32 (1-2):67-76.
13. Vaseva AV, Blake DR, Gilbert TS. Hostetter G. KRAS Suppression-Induced Degradation of MYC Is Antagonized by a MEK5-ERK5 Compensatory Mechanism. Cancer Cell. 2018;34 (5): 807-822.e7.
14. Brescia P, Ortensi B, Fornasari L, Levi D, Broggi G. CD133 is essential for glioblastoma stem cell maintenance. Stem cells (Dayton, Ohio). 2013;31 (5): 857-69.
15. Bhatt AB, Patel S, Matossian MD, Ucar DA, Miele L. Molecular Mechanisms of Epithelial to Mesenchymal Transition Regulated by ERK5 Signaling. Biomolecules. 2021;11(2).
16. Hoang VT, Yan TJ, Cavanaugh JE, Flaherty PT, Beckman BS. Oncogenic signaling of MEK5-ERK5. Cancer letters. 2017; 392: 51-59.
17. Hoang VT, Matossian MD, Ucar DA, Elliott S, La J. ERK5 Is required for tumor growth and maintenance through regulation of the extracellular matrix in triple negative breast cancer. Frontiers in Oncology. 2020;10(1164).
18. Gusev Y, Bhuvaneshwar K, Song L, Zenklusen JC, Fine H. The REMBRANDT study, a large collection of genomic data from brain cancer patients. Scientific Data. 2018; 5(1):180158.
19. An Z, Aksoy O, Zheng T, Fan QW, Weiss WA. Epidermal growth factor receptor and EGFRvIII in glioblastoma: signaling pathways and targeted therapies. Oncogene. 2018; 37(12):1561-1575.
20. Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: Divergent roles of isoforms, modes of activation and therapeutic targeting. Nat Rev Cancer. 2015; 15 (1):7-24.
21. Diaz Miqueli A, Rolff J, Lemm M, Fichtner I, Perez R. Radiosensitisation of U87MG brain tumours by anti-epidermal growth factor receptor monoclonal antibodies. British J Cancer. 2009; 100 (6): 950-958.
22. Pore N, Liu S, Haas-Kogan DA, O'Rourke DM, Maity A. PTEN mutation and epidermal growth factor receptor activation regulate vascular endothelial growth factor (VEGF) mRNA expression in human glioblastoma cells by transactivating the proximal VEGF promoter. Cancer research. 2003;63 (1): 236-41.
23. Tetsu O, McCormick F. ETS-targeted therapy: Can it substitute for MEK inhibitors?. Clinical and Translational Medicine. 2017; 6 (1):16.
24. Wu J, Cui H, Zhu Z, Wang L. MicroRNA-200b-3p

suppresses epithelial-mesenchymal transition and inhibits tumor growth of glioma through down-regulation of ERK5. Biochemical and biophysical research communications. 2016; 478(3):1158-64.

25. Tubita A, Lombardi Z, Tusa I, Dello Sbarba P, Rovida E. Beyond kinase activity: erk5 nucleo-cytoplasmic shuttling as a novel target for anticancer therapy. Int J Mol Sci. 2020; 21 (3):938.

26. Matossian MD, Burks HE, Elliott S, Hoang VT, Bowles AC. Panobinostat suppresses the mesenchymal phenotype in a novel claudin-low triple negative patient-derived breast cancer model. Oncoscience. 2018; 5(3-4): 99-108.

27. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 2011;75(1): 50-83.