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NVP-BEZ235 or JAKi Treatment leads to decreased survival of examined GBM and BBC cells

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ABSTRACT

Cancer cells almost universally harbor constitutively active Phosphatidylinositol-3 Kinase (PI3K) Pathway activity via mutation of key signaling components and/or epigenetic mechanisms. Scores of PI3K Pathway inhibitors are currently under investigation as putative chemotherapeutics. However, feedback and stem cell mechanisms induced by PI3K Pathway inhibition can lead to reduced treatment efficacy. To address therapeutic barriers, we examined whether JAKi would reduce stem gene expression in a setting of PI3K Pathway inhibition in order to improve treatment efficacy. We targeted the PI3K Pathway with NVP-BEZ235 (dual PI3K and mTOR inhibitor) in combination with the Janus Kinase inhibitor JAKi in glioblastoma (GBM) and basal-like breast cancer (BBC) cell lines. We examined growth, gene expression, and apoptosis in cells treated with NVP-BEZ235 and/or JAKi. Growth and recovery assays showed no significant impact of dual treatment with NVP-BEZ235/JAKi compared to NVP-BEZ235 treatment alone. Gene expression and flow cytometry revealed that single and dual treatments induced apoptosis. Stem gene expression was retained in dual NVP-BEZ235/JAKi treatment samples. Future in vivo studies may give further insight into the impact of combined NVP-BEZ235/JAKi treatment in GBM and BBC.

Abbreviations

PI3K: Phosphatidylinositol 3 Kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PIP3: phosphatidylinositol 3,4,5-trisphosphate
AKT: protein kinase B
FOXO: Forkhead box subfamily O
ES: embryonic stem
GBM: glioblastoma multiforme
BBC: Basal breast cancer
HER2: Human Epidermal Growth Factor Receptor 2
OCT4: Octamer-binding Transcription factor 4
SOX2: SRY-Box Transcription Factor 2
MEM: minimal essential media
DMEM: Dulbecco's Modified Eagle Medium
RPMI: Roswell Park Memorial Institute (RPMI) 1640 Medium
FBS: fetal bovine serum
PBS: Phosphate buffered saline

NANOG: Nanog Homeobox
ALPL: Alkaline Phosphatase Liver
TRAIL: TNF-related apoptosis-inducing ligand
BIM: Bcl-2-like protein 11
FAS: TNF Receptor Superfamily, Member 6
STAT3: Signal transducer and activator of transcription 3
JAK2: Janus Kinase 2
mTOR: mammalian target of rapamycin
EGFR: Epidermal Growth Factor Receptor
RTK: Receptor tyrosine kinase
IBC: Institutional Biosafety Committee

Introduction

In 2008 Weinberg and colleagues discovered that certain poorly-differentiated aggressive cancers such as glioblastoma multiforme (GBM) and basal-like breast cancer (BBC) harbor an embryonic stem cell-like gene expression signature [1]. These cancers express stem genes

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such as *OCT4* (Octamer-binding Transcription Factor 4), *SOX2* (*SRY-Box Transcription Factor 2*) and *NANOG* (*Nanog Homeobox*) [1-4]. Stem genes contribute to poor prognosis in GBM and BBC [5,6]. Of note, we and others found that like embryonic stem cells (ESC), the PI3K (Phosphatidylinositol 3-kinase) Pathway is fundamentally rewired in GBM and BBC [7-11]. Transcription factor FOXO1 (Forkhead box O 1) was functional despite constitutively active PI3K (against the paradigm) in GBM and BBC [7]. Furthermore, FOXO1 promoted stem gene expression in GBM and BBC cancer cells via a similar mechanism described in ESC [7, 10]. PI3K inhibitor treatment induced stem gene expression in GBM and BBC cells [7,8].

GBM is a prevalent type of tumor of the central nervous system [12, 13]. GBM has high metastatic abilities and an aggressive phenotype which poses a challenge on surgical removal, giving patients an average 15 months of survival [14,15]. Breast cancer can be classified based on gene expression signatures into distinct subtypes such as luminal A, luminal B, BBC, HER2 positive and normal-like [16-19]. BBC comprises approximately 10–17% of breast cancers in the United States and has characteristics in common with myoepithelial cells of the breast [20-22]. Frequently, BBC lacks expression of estrogen, progesterone and HER2 receptors, making these cancers unresponsive to hormonal-based therapies [23,24]. BBC is associated with poor prognosis [25-27]. In addition to harboring stem-like signatures, GBM and BBC are associated with self-renewal, metastasis, and chemotherapeutic resistance [1]. Despite therapeutic advances, targeting the bulk of tumorigenic cells does not exclude patients from future metastasis and relapse [28-30]. Targeting pathways that drive the stem signatures in GBM and BBC may ultimately decrease patient relapse [31-33].

The PI3K Pathway is almost universally activated in cancer by mutations to promote growth and survival [34,35]. Endogenously, the PI3K Pathway is activated by growth factors binding to receptor tyrosine kinases (RTKs). Active PI3K converts phosphatidylinositol-4,5, bisphosphate (PIP₂) to phosphatidylinositol-3,4,5 trisphosphate (PIP₃) [36]. Tumor suppressor *PTEN* encodes a phosphatase that converts PIP₃ to PIP₂ to down-regulate this pathway [37-39]. Second messenger PIP₃ membrane-recruits and activates AKT which phosphorylates FOXO transcription factors (FOXO -1, -3, and -4) among other substrates [40]. FOXO transcription factors regulate metabolism, apoptosis, tumor suppression and cellular differentiation [41-43]. FOXO transcription factors also promote stem characteristics in embryonic stem (ES), BBC, and GBM cells in part by activating *OCT4* gene expression [7,10]. In GBM and BBC, PI3K Pathway genes are characteristically mutated to activate signaling output. *PTEN* is commonly mutated to an inactive form, whereas the RTK gene *EGFR* (*Epidermal Growth Factor*) is mutated to encode an active form leading to increased growth and survival [44-46].

Given the central role of the PI3K pathway in driving tumor formation and progression, it is a common chemotherapeutic target [34, 47-49]. PI3K pathway targeted therapies in GBM and BBC have improved in selectivity and stability while also decreasing in toxicity [50,51]. EGFR inhibitor erlotinib slowed GBM progression in a fraction of patients [52-54]. An enhanced PI3K inhibitor that targets both PI3K and mTOR (mammalian target of rapamycin), NVP-BEZ235 improved survival outcomes and elicited advanced antitumor effects in GBM [55-58]. However, we and others have observed that targeting the PI3K pathway leads to the induction of stem genes in GBM and BBC cells, potentially promoting cancer recurrence [7,8].

Stem gene expression in GBM and BBC is activated by numerous mechanisms including the Janus Kinase 1/Janus Kinase 2 (JAK1/2) and STAT3 (Signal Transducer and Activator of Transcription 3) Pathway [59,60]. STAT3 is endogenously activated by JAK1/2 upon cytokine binding to cognate receptors. JAK1/2 phosphorylates STAT3 on tyrosine 705, leading to homodimerization and regulation of target genes in the nucleus [61]. The JAK/STAT3 Pathway becomes constitutively active in cancer cells to accelerate tumor progression [61]. In GBM and BBC, activating mutations in *JAK1*, *JAK2* and *EGFR* lead to constitutively

active STAT3 [46,62-65]. Increased cytokine signaling also contributes to STAT3 activation in these cancers [66-69]. Active STAT3 during tumorigenesis contributes to proliferation and hinders apoptosis [70-72]. STAT3 sustains cancer stem cells and promotes metastasis [73-78]. In pluripotent stem cells JAK1 activates STAT3, which in turn directly binds to the *OCT4* promoter to induce transcription [59]. Reduced STAT3 expression in pluripotent stem cells led to a loss in stem cell marker alkaline phosphatase [59]. Based on this, we reasoned that JAK inhibition may reduce stem characteristics in GBM and BBC cells.

JAK inhibitors have proved effective at decreasing invasiveness and tumorigenesis in GBM and BBC in preclinical studies [79-81]. Pacritinib increased survival in GBM orthotopic xenograft studies [82]. Ruxolitinib decreased migration and colony formation in GBM cells [79]. AZD1480 reduced GBM xenograft growth [83]. Similarly, JAK inhibition with ruxolitinib was examined as a therapeutic for BBC with limited success [84]. Although ruxolitinib was able to diminish STAT3 activation in BBC, it was not efficacious as a single agent therapy [84].

One barrier to effectively employing PI3K targeted therapies in GBM and BBC is the induction of stem genes, which may ultimately promote resistance and/or recurrence [7,8]. We hypothesized that targeting the PI3K Pathway (to suppress growth and survival) in combination with JAKi (to suppress growth and stem gene expression) may have a combinatorial impact on GBM and BBC.

Materials and methods

Cell culture

Cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA) and grown under standard conditions (5% CO₂, 10% FBS (fetal bovine serum), with 5% antibiotic/antimycotic (Thermo Fisher, Waltham, MA)). Cell lines were tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel Switzerland, cat: LT07-218); all experiments were done with mycoplasma negative cells. U87MG cells were propagated in MEM (Minimal Essential Medium). DBTRG and BT549 cells were propagated in RPMI (Roswell Park Memorial Institute 1640 Medium). LN18, U118MG, A172, MDA-MB-468 and LN229 cells were propagated in DMEM (Dulbecco's Modified Eagle Medium).

Drug treatments

Cells were plated at a density of 15,000 cells per mL and were treated for indicated number of days with indicated drugs for growth assays. Recovery assays included indicated drug treatments added to cells plated at 2700 cells per mL. Recovery assay samples had fresh media added after six days of drug treatment; these samples were allowed to recover for six days in fresh media before staining to assess cellular growth after treatment. Treatments were investigated in triplicate (in numerous independent experiments) and stained with crystal violet. Plates were aspirated of media then washed with 0.5 ml of 1x phosphate buffered saline (PBS) before being stained with 0.5 ml of crystal violet stain (0.5% crystal violet in buffered formalin) and incubated for 15 min. The stain was aspirated, and wells were washed 3 times with 0.5 ml of 1x PBS. After collections were completed, crystal violet-stained plates were solubilized using 0.5 ml on each well of 10% acetic acid and placed on shaker for 1 hour. Solubilized samples were transferred to 96 well plates and quantified on a spectrophotometer at 590 nm. Quantified plates were analyzed with a Tukey Test on vassarstats.net for statistical significance. Error bars were added using the standard error of the collection readouts. NVP-BEZ235 was purchased from Sigma (Saint Louis, MO), and utilized at a final concentration of 50 nM in growth and recovery experiments. JAKi was purchased from Sigma and used at a final concentration of 2 μM. NVP-BEZ235 and JAKi were dissolved in DMSO.

Western blot

Cell lines were treated with 50 nM NVP-BEZ235, 2 μ M JAKi and/or DMSO (solvent) for 24 h. Total protein was obtained by rinsing cells with 1XPBS (phosphate buffered saline) followed by direct lysis in 2x sample buffer (125 mM Tris-HCL at pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% glycerol, 0.05% bromophenol blue, 8 M urea); 2x sample buffer was added to each well and cells scraped with a cell scraper. The lysate was collected from each well, placed into 1.5 mL microcentrifuge tubes and heated for 10 min at 95 °C in a dry-bath heat block. Protein lysates were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1 hour. The protein was then transferred onto a polyvinylidene fluoride (PVDF) membrane for an hour and 30 min then blocked in a 5% milk solution (Carnation powdered milk, 1X Tris-buffered saline with Tween 20 (TBST) for an hour. The membrane was incubated with indicated primary antibody overnight at 4 °C then, washed for 20 min with TBST in 5-minute intervals. The blot was then incubated with secondary antibodies for 1.5 h. The membrane was washed again for 20 min in 5-minute intervals and allowed to develop using SuperSignal West Dura Extended Duration Substrate luminol solution (Pierce Biotechnology, Waltham, MA) for 5 min. A Bio Rad ChemDoc XRS+ Molecular Imager was utilized for protein detection (Bio Rad Hercules, CA). Densitometry was performed using Image Lab Software (Bio Rad Hercules, CA). Relative expression was calculated using total band volume of a protein of interest divided by the GAPDH total band volume for the protein sample. Band densities were depicted relative to the DMSO solvent control. Antibodies were obtained from Cell Signaling Technologies (Danvers, MA): total STAT3 (9139T), phospho STAT3 tyrosine 705 (9145T), and phospho AKT serine 473 (9271S) and total AKT (4691S). GAPDH (G-9) was obtained from Santa Cruz Biotechnology Inc., Dallas, TX.

Quantitative real time PCR

Total RNA was prepared using the Qiagen RNeasy kit (Hilden, Germany), which was then used to generate cDNA with Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). Samples (cDNAs) were analyzed using (Power SYBR Green Master Mix, Applied Biosystems, Foster City, CA) and the Illumina Eco Real-time system (San Diego, CA). Expression levels were normalized to *GAPDH* in gene expression experiments and calculated using $2^{-\Delta\Delta CT}$ method [85]. Primer sequences are detailed in supplemental Table S1. All drug treatments utilized 50 nM NVP-BEZ235 and 2 μ M JAKi. Time points for RNA collection were optimized for each cell line. U87MG and LN229 RNA lysates were collected after five days of drug treatment. BT549 RNA lysates for stem genes were collected after five days of drug treatment. BT549 RNA lysates for apoptosis were collected after 24 h. MDA-MB-468 RNA lysates were collected after three days of drug treatment.

Flow cytometry

All cell lines were treated with 50 nM NVP-BEZ235, 2 μ M JAKi and/or DMSO. U87MG cells were treated with drug for five days before assessing apoptosis, whereas LN229, BT549 and MDA-MB-468 cells were treated with drug for three days before assessing apoptosis. Growth medium was aspirated after indicated treatment. Cells were incubated with 2 mL of 0.25% trypsin each for 5 min at room temperature. 1.5 mL of each plate was transferred into 1.7 mL microcentrifuge tubes and centrifuged at Gx0.2 for 10 min. Trypsin supernatant was aspirated, cells were resuspended with 1xPBS and then centrifuged at Gx0.2 for 10 min. 1xPBS supernatant was aspirated and cells in microcentrifuge tubes were then resuspended with dilute binding buffer (Molecular Probes FITC Annexin V/Dead Cell Apoptosis Kit from Thermo Fisher, Waltham, MA). 100 μ L of each sample diluted with binding buffer were added to a fresh microcentrifuge tube. Each tube had 2 μ L of Annexin V FITC and 1 μ L

of PI added and incubated for 15 min in the dark. 400 μ L of binding buffer was added after the 15-minute incubation and cells were sorted by BD FACS Celesta flow cytometer (BD Biosciences, San Jose, CA).

Results

Treatment with NVP-BEZ235 or JAKi led to a reduction in GBM and BBC cells

To assess the impact of combined PI3K pathway and JAK inhibition on cancer cell growth and survival, we treated U87MG GBM, LN229 GBM, BT549 BBC, and MDA-MB-468 BBC cancer cell lines with vehicle (DMSO), 50 nM NVP-BEZ235, and/or 2 μ M JAKi (Fig. 1A-D) for five days. Plates were stained with crystal violet at indicated time points and data were analyzed by ANOVA with Tukey HSD Test. In the U87MG cell line on day 5, each treatment (JAKi, NVP-BEZ235 and combination of JAKi with NVP-BEZ235) was found to have reduced cell numbers compared to the DMSO vehicle ($P < 0.01$). JAKi single treatment had a significantly lower growth inhibition ($P < 0.01$) when compared to single NVP-BEZ235 and double treatment of JAKi/NVP-BEZ235. However, there was no significant difference between the single NVP-BEZ235 treatment and the JAKi/NVP-BEZ235 double treatment for cell growth. In the BT549, LN229 and MDA-MB-468 cell lines on day five, each treatment (JAKi, NVP-BEZ235 and combination of JAKi with NVP-BEZ235) was found to negatively impact growth compared to the DMSO vehicle ($P < 0.01$). However, there was no significant difference between JAKi, NVP-BEZ235, and JAKi/NVP-BEZ235 treatments in growth assays (Fig. 1A-D).

Protein lysates were utilized to assess inhibition of STAT3 (via 2 μ M JAKi treatment) and AKT activation (via PI3K/mTOR inhibition, 50 nM NVP-BEZ235 treatment) with GAPDH as a loading control using western blot and densitometry analyses (Fig. 2A-F). Lysates collected from NVP-BEZ235 drug-treated samples had reduced P-AKT (S473), whereas extracts collected from the JAKi drug-treated cells showed reduced P-STAT3 (Y705).

GBM cells showed reduced cell number in recovery assays with PI3K or JAK inhibition

Given the similarities between the single NVP-BEZ235 combined NVP-BEZ235/JAKi treatments in growth assays, we sought to further investigate the phenotype by performing recovery assays. We treated indicated cell lines with vehicle (DMSO), NVP-BEZ235 alone (50 nM), JAKi alone (2 μ M) or combined NVP-BEZ235/JAKi for six days. After drug treatment, we added fresh media and allowed the cells to recover for six days to determine differences in cell survival. The recovery assays would allow cytostatic cells to grow back, highlighting potential differences between cytotoxic and cytostatic impacts. Cells were stained with crystal violet as described in the *Materials and Methods* to assess cell numbers following treatment and recovery. We observed significantly decreased cell numbers with single and double treatment samples (NVP-BEZ235 and/or JAKi) based on ANOVA with Tukey HSD Test with reference to vehicle (DMSO) controls in all cell lines examined: GBM cell lines U87MG, DBTRG, LN18, LN229 and U118MG, as well as BBC cell lines BT549 and MDA-MB-468 (Fig. 3A-G). However, there was no significant change between NVP-BEZ235 and NVP-BEZ235/JAKi treatments in recovery assays for any of the cell lines examined by Tukey HSD Test (Fig. 3A-G).

Treatment with NVP-BEZ235 or JAKi led to increased apoptosis in examined GBM and BBC cells

Gene expression analyses were performed to investigate mechanisms driving cell loss in U87MG, LN229, BT549 and MDA-MB-468 samples treated with the NVP-BEZ235 and/or JAKi. We initially hypothesized that the dual treatment would potentially cause a shift to reroute FOXO

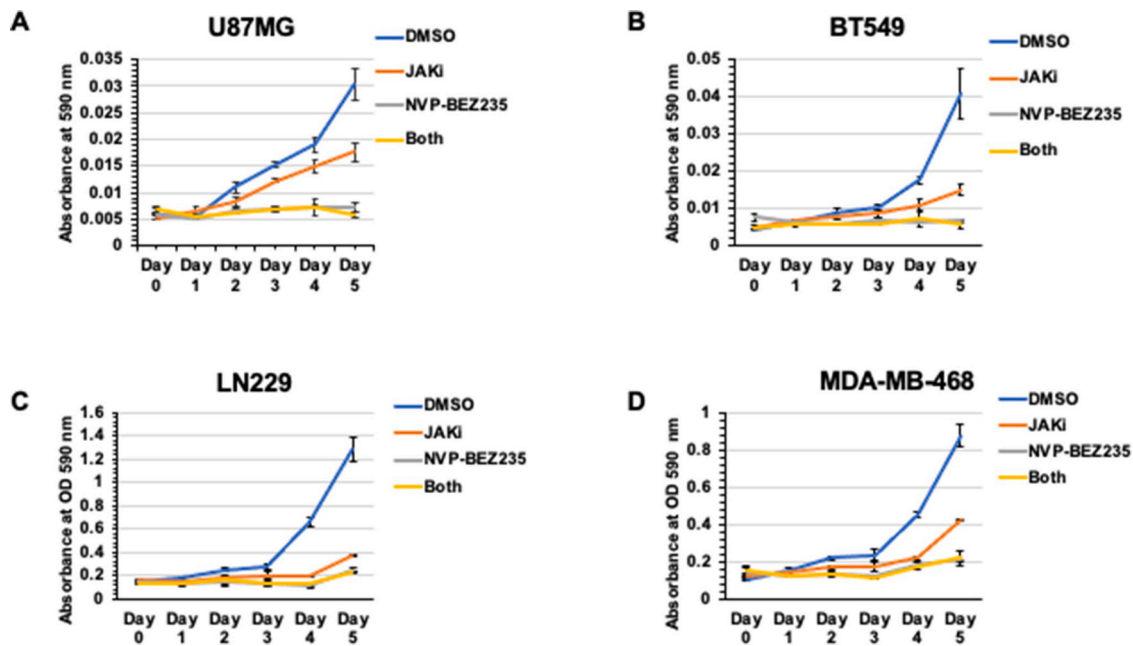


Fig. 1. NVP-BE2235 or JAKi reduced Growth in Examined GBM and BBC Cell Lines **A.** Growth assays with U87MG cells treated with 50 nM NVP-BE2235 and/or 2 μ M JAKi. All treatments significantly impacted growth compared to DMSO control with no increased impact from dual NVP-BE2235/JAKi treatment (compared to single NVP-BE2235 alone) based on ANOVA with Tukey HSD Test. JAKi treatment impacted growth significantly less than NVP-BE2235. **B-D.** Growth assays with BT549, LN229 and MDA-MB-468 cells treated with 50 nM NVP-BE2235 and/or 2 μ M JAKi. All treatments significantly impacted growth compared to DMSO control. JAKi treatment was not significantly different compared to NVP-BE2235 or NVP-BE2235/JAKi based on ANOVA with Tukey HSD Test.

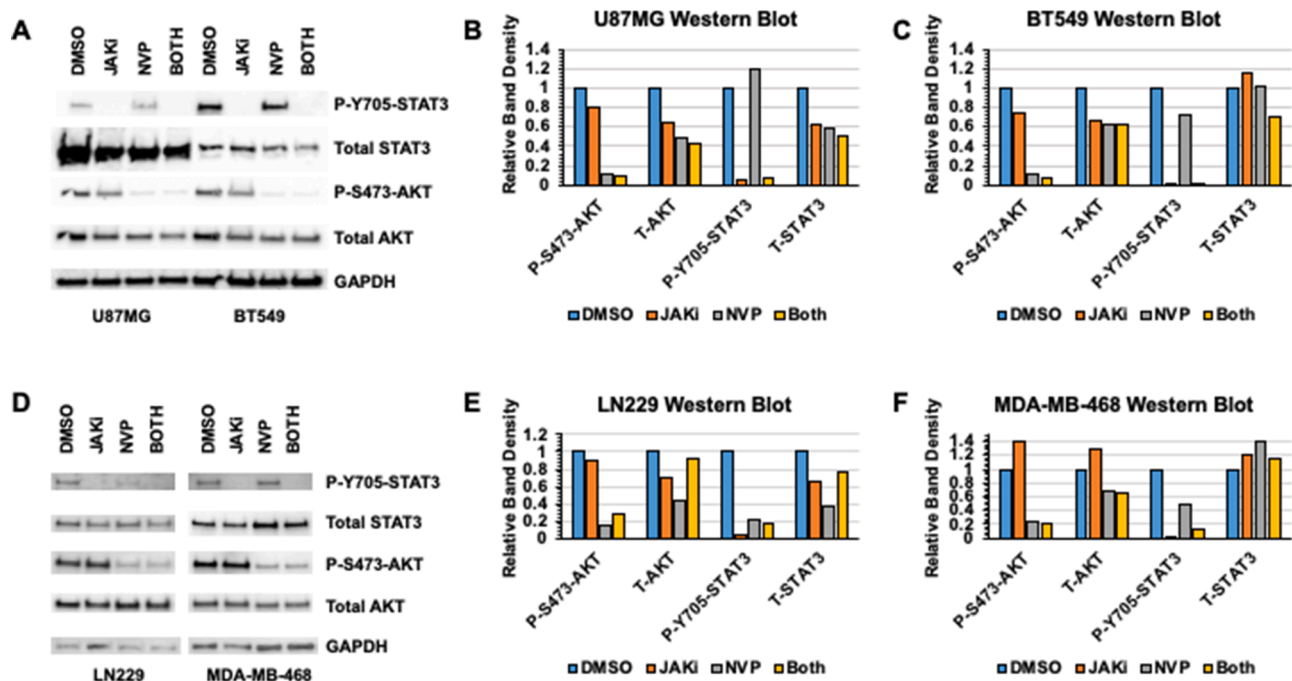


Fig. 2. Western Blot Analysis for NVP-BE2235 or JAKi Treatments **A.** Total protein lysates were collected 24 h post NVP-BE2235 (50 nM) and/or JAKi (2 μ M) treatment in U87MG and BT549 cells and examined by western blot analysis with indicated antibodies. **B-C.** Densitometry was performed using Image Lab Software (Bio-Rad) for U87MG and BT549 western blot data as described in the *Materials and Methods*. P-AKT (S473) was reduced in NVP-BE2235 treatment samples, whereas P-STAT3 (Y705) was reduced in JAKi treatment samples. **D.** Total protein lysates were collected 24 h post NVP-BE2235 (50 nM) and/or JAKi (2 μ M) treatment in LN229 and MDA-MB-468 cells and examined by western blot analysis with indicated antibodies. **E-F.** Densitometry was performed using Image Lab Software (Bio-Rad) for LN229 and MDA-MB-468 western blot data. P-AKT (S473) was reduced in NVP-BE2235 treatment samples, whereas P-STAT3 (Y705) was reduced in JAKi treatment samples.

factors to induce apoptotic genes at the expense of stem genes. Stem factor *OCT4* is activated in part by STAT3 [86,87]. FOXO1 was also previously shown to activate *OCT4* gene expression in stem cells as well

as at least some GBM and BBC cancer cell lines [7,10]. We tracked apoptotic genes that FOXO factors were known to induce: *TRAIL*, *BIM*, and *FAS*[88]. We found evidence of apoptotic gene induction by each

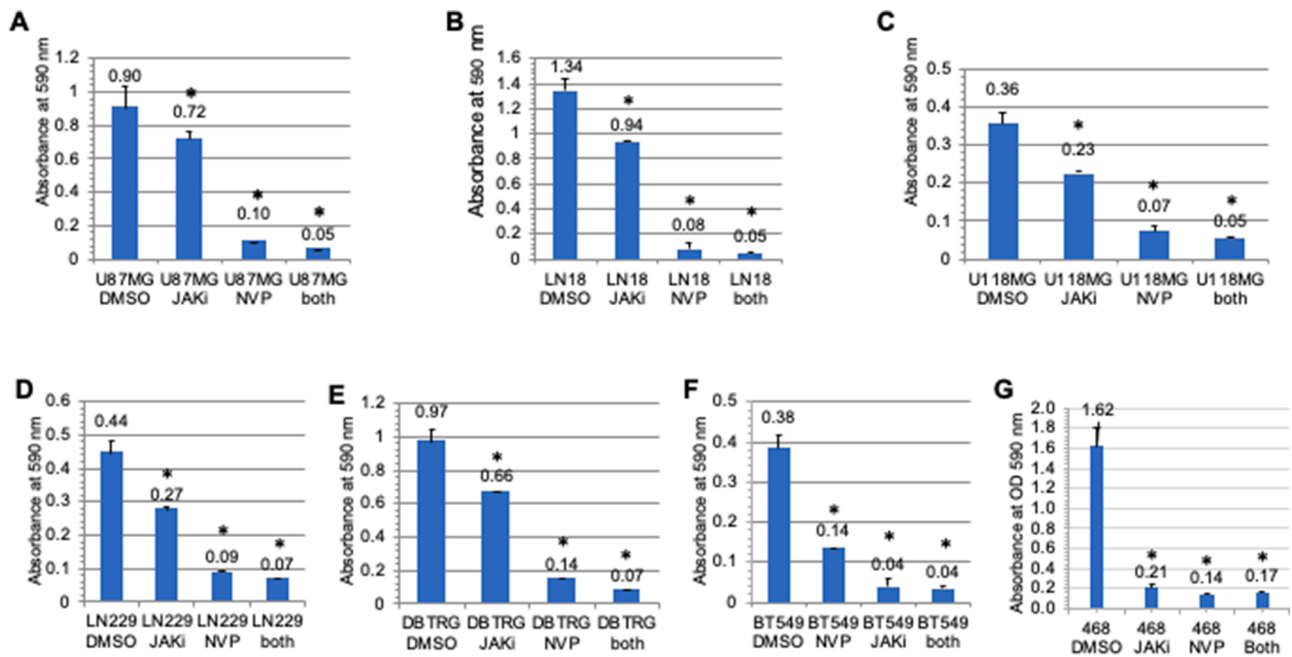


Fig. 3. NVP-BE2235 or JAKi treatment reduced cell numbers in recovery assays. Recovery assays were performed with GBM and BBC cell lines in which cells were treated with indicated drugs for six days and were then allowed to recover in complete media without drug for six days. **A-G.** All drug treatments in each examined cell line significantly impacted cell numbers compared to DMSO control (denoted *), with no increased impact from dual NVP-BE2235/JAKi treatment compared to NVP-BE2235 alone based on ANOVA with Tukey HSD Test. NVP-BE2235 was abbreviated NVP. MDA-MB-468 was abbreviated 468.

drug treatment in each of the cell lines examined compared to DMSO controls, except for JAKi and combined NVP-BE2235/JAKi treatments in MDA-MB-468 (Figs. 4A-B and S1 A-B). In contrast to our hypothesis, all examined cell lines retained *OCT4* expression as a stem marker in combination NVP-BE2235/JAKi treated samples (Figs. 4C-D and S1

C-D). Some of the cell lines retained expression of additional markers such as *SOX2*, *NANOG* and *ALPL* (*Alkaline Phosphatase Biomineralization Associated*), (Figs. 4C-D and S1 C-D). Therefore, the combined NVP-BE2235/JAKi treatment succeeded at inducing apoptotic genes but failed to suppress stem gene expression.

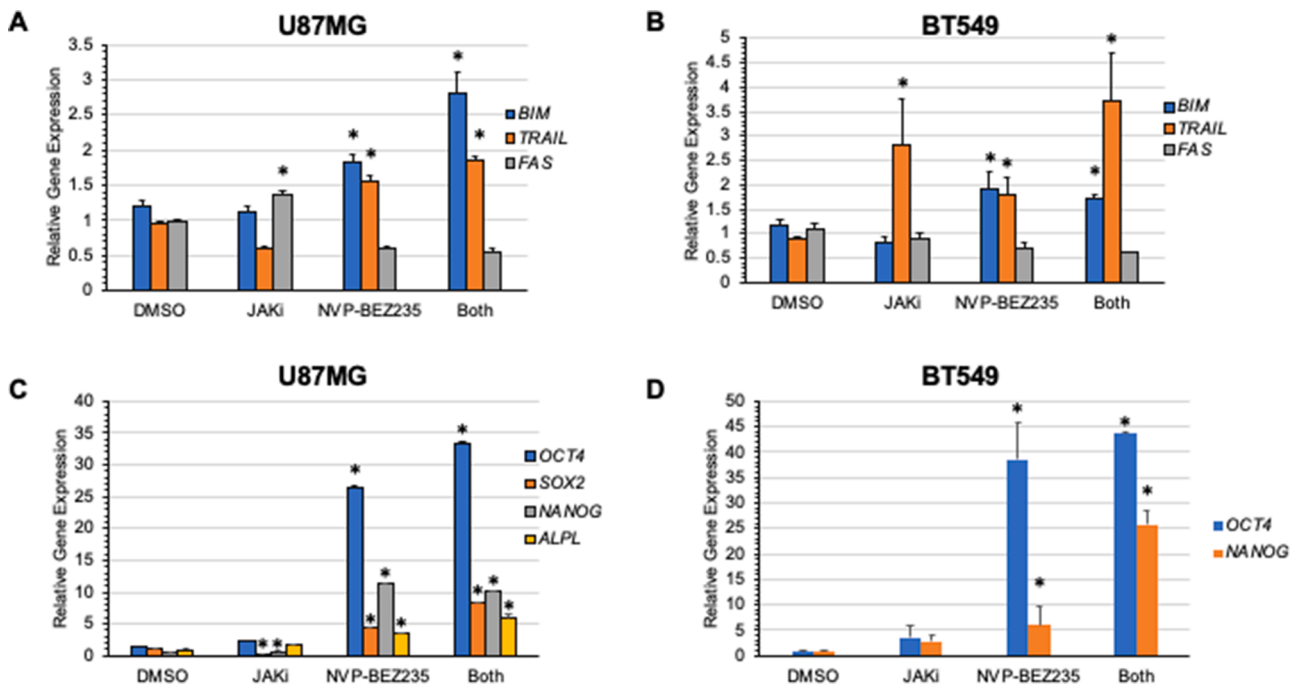


Fig. 4. Treatment of examined GBM or BBC cells with Dual PI3K Inhibitor NVP-BE2235 and/or JAKi Induced Apoptotic Gene Expression. **A-B.** Gene expression of FOXO-induced apoptotic targets (determined by qRT-PCR relative to *GAPDH* control) in U87MG and BT549 cells. NVP-BE2235 treatment led to induction of *BIM* and *TRAIL*. **C.** Expression of stem genes from drug treatment samples by qRT-PCR (relative to *GAPDH* control). Stem genes *OCT4*, *SOX2*, *NANOG* and *ALPL* were induced in U87MG NVP-BE2235 treatment samples with or without JAKi. Single agent treatment with JAKi led to reduced *SOX2* and *NANOG*. **D.** Expression of *OCT4* and *NANOG* in BT549 cells with indicated drug treatments. *OCT4* and *NANOG* remained induced with the combined NVP-BE2235/JAKi treatment based on ANOVA with Tukey HSD Test (denoted *).

To further examine apoptotic induction by NVP-BEZ235 and/or JAKi treatment, we performed flow cytometry. The marker for early apoptosis annexin-V conjugated with green fluorescent dye FITC was used to bind to externally exposed phospholipid phosphatidylserine thereby detecting membrane depolarization [89]. A DNA intercalating agent and late marker of apoptosis propidium iodide (PI) was used to detect cell permeability [90]. Cells stained with annexin-V FITC and/or PI were counted as apoptotic. Treatment with NVP-BEZ235 alone or the combination of NVP-BEZ235/JAKi led to similar levels of apoptosis in BT549 cells compared to DMSO controls (Fig. 5A-E). JAKi alone, NVP-BEZ235 alone, and combined NVP-BEZ235/JAKi treatments (compared to DMSO control) led to apoptotic induction in U87MG, LN229 and MDA-MD-468 cell lines based on ANOVA with Tukey HSD Test (Fig. 5E).

Conclusion

The PI3K Pathway is almost universally constitutively active in GBM and BBC due to pathway mutations [37,44,46]. Activation of the PI3K Pathway drives tumor formation, progression, and metastasis, making it an ideal target for therapeutic intervention [34,36,47]. The use of PI3K inhibitors to treat GBM and BBC was previously shown to significantly induce cell death [91-93]. Cell cycle arrested remnants could develop resistance to treatments to become metastatic [94]. The 50 nM dose of NVP-BEZ235 employed in this work was utilized in clinical settings and pre-clinical studies [55,56]. Similar doses of NVP-BEZ235 examined in GBM cell lines (U87MG, U251, T98G and SGH44) induced G1 cell cycle arrest and apoptosis [55]. NVP-BEZ235 treatment also reduced chemoresistance to temozolomide in GBM models and enhanced radiosensitivity of GBM stem cells [55,56]. Low doses of NVP-BEZ235 (10-100 nM) led to either growth arrest or cell death in BBC cell lines [95,96]. STAT3 inhibition led to a decrease in temozolomide resistance in GBM [97]. Likewise, inhibition of JAK/STAT led to decreased invasiveness in GBM and BBC [83,84]. Our study employed 2 μ M JAKi based on work by

Marotta et al. in a BBC setting [78]. Clinical trials are underway to examine the impact of JAK inhibition as a therapeutic for GBM and BBC [79,84].

Although the combined treatment of NVP-BEZ235/JAKi had a limited impact in our study, others have found potent efficacies in acute lymphoblastic leukemia and B cell malignancies [98-100]. Signaling redundancies that induce stem characteristics may underpin the lack of efficacy in GBM and BBC cells.

Examined GBM and BBC cells retained expression of stem genes in dual treatment (NVP-BEZ235 and JAKi) samples. Therefore, inhibition of JAKi was not sufficient to block stem gene expression in the NVP-BEZ235 treated samples (Fig. 4C-D and S1 C-D). The impact of STAT3 on stem gene expression is context dependent [59,101,102]. It remains to be determined if the increase in stem gene expression with NVP-BEZ235 treatment resulted from the survival of cells that express stem genes (such as cancer stem cells surviving and non-cancer stem cells undergoing apoptosis), leading to an enrichment in cancer stem cells. Alternatively, JAK-independent mechanisms may drive stem gene expression in this setting or the cells that express these stem genes may be resistant to drug treatment (perhaps via drug efflux). The timing of drug treatment may also contribute to therapeutic efficacy. Treatment of GBM with NVP-BEZ235 for one hour prior to with ionizing radiation strongly enhanced radiosensitivity [103]. In contrast, a 24-hour NVP-BEZ235 treatment prior to IR treatment led to G1 arrest and exhibited less DNA damage; there was no enhancement of IR sensitivity with the 24-hour treatment [103]. Delineating molecular mechanisms that drive stem characteristics and therapeutic resistance is key to improving chemotherapeutic efficacy for GBM and BBC.

Ethics approval

Work was performed with Institutional Biosafety Committee approval from the University of Texas Rio Grande Valley: Registration

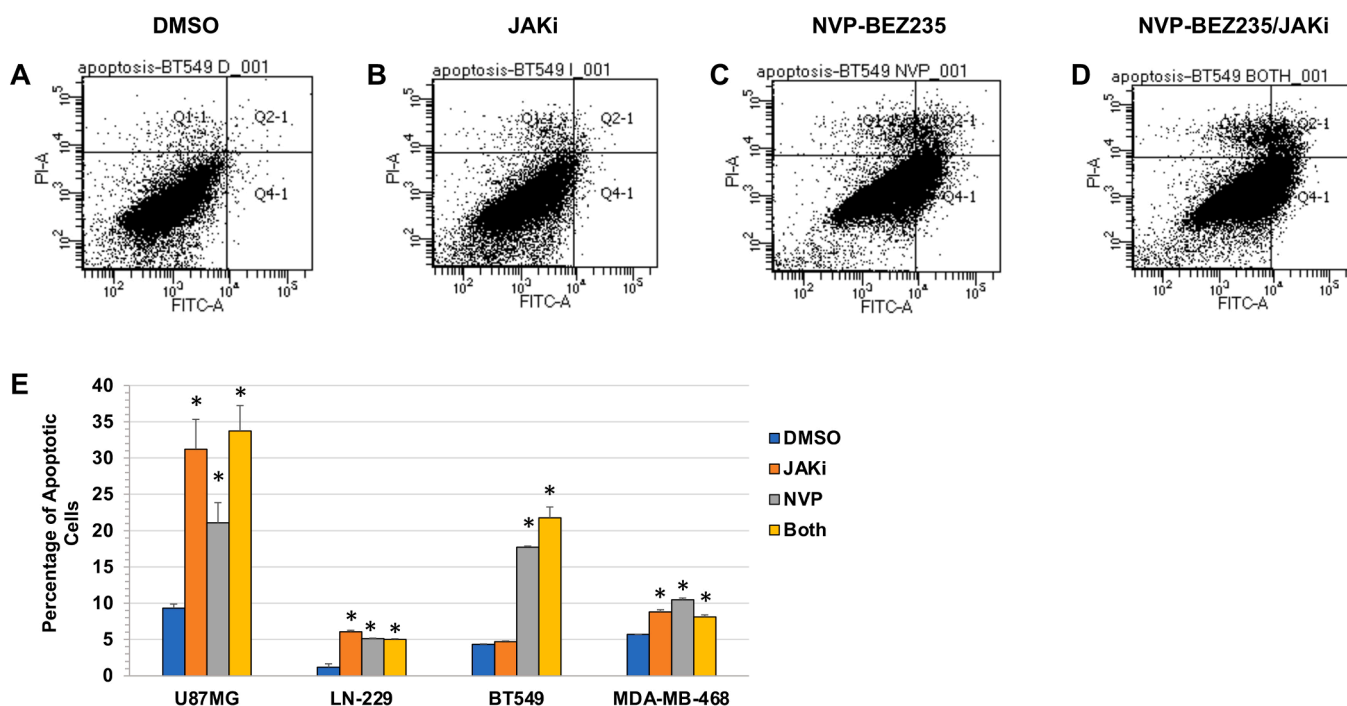


Fig. 5. Flow cytometric analyses revealed apoptosis induction by NVP-BEZ235 and/or JAKi in examined GBM and BBC cell lines. Cells treated with indicated drugs were stained with AnnexinV-FITC and PI and were examined by flow cytometric analyses. A-D. Treatment of BT549 cells with NVP-BEZ235 alone or combined NVP-BEZ235/JAKi led to increased AnnexinV-FITC and PI staining. E. The percentage of apoptotic cells was determined by flow cytometric analyses for indicated drug-treated cell lines. AnnexinV-FITC and/or PI staining was counted as positive for apoptosis. Treatment with JAKi alone, or combined NVP-BEZ235/JAKi led to significantly increased apoptosis compared to DMSO controls in U87MG, LN229 and MDA-MB-468 cells, whereas NVP-BEZ235 alone or in combination with JAKi led to apoptosis in BT549 cells based on ANOVA with Tukey HSD Test (denoted *).

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Availability of data and materials

All cell lines and additional data prepared from this work are available upon request.

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Declaration of Competing Interests

The authors declare that they have no competing interests.

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Supplementary materials

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