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Investigating the Efficacy of JAK2/STAT3 and PI3K/mTOR Inhibitory Therapy on Glioblastoma and Basal Breast Cancer Cell Lines

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INVESTIGATING THE EFFICACY OF JAK/STAT3 AND PI3K/MTOR INHIBITORY THERAPY ON GLIOBLASTOMA AND BASAL BREAST CANCER CELL LINES

A Thesis

by

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ABSTRACT

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JAK/STAT3 and PI3K/mTOR pathways are upregulated in cancer cells. STAT3 promotes a positive stem program feedback loop. PI3K inhibits the ability of FOXOs to perform tumor suppressor roles whereas mTOR promotes tumorigenic advancement. GBM and BBC are aggressive cancers that harbor stem cell signatures and are resistant to chemotherapy. The effects of combination therapy inhibiting JAK/STAT3 and PI3K/mTOR were examined. Our collected data indicates that dual inhibition induced apoptosis, but surprisingly it also increased the stem cell signature. Western blotting indicated that the proteins were effectively inhibited by the drug treatments. Cell growth was tracked through a calorimetric assay. Flow cytometry analyses indicated a significantly higher apoptotic outcome with the double treatment. Gene expression was assessed by qRT-PCR in which: *BIM*, *TRAIL*, *OCT4*, *SOX2*, *ALPL*, and *NANOG* were induced with the double treatment. Future isolation of the remaining stem cells will help elucidate their intricacies to target them effectively.

DEDICATION

The completion of my master studies was fueled by the love and support of my family. My siblings Cristopher and Jonathan for the much-needed laughs that got me through tough times. My sister Getsemaní for always believing in me and being a role model whose shoes I will forever aim to fill. To my parents Lupita and Javier for teaching me the importance of an education and giving me their unwavering support to go far in my academic career. Special thanks to my mother who has always been by my side providing me with emotional support as well as being a role model for high morale, work ethic, and determination.

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CHAPTER I

INTRODUCTION

Breast cancer is one of the most aggressive forms of cancer for women around the world. Through the use of DNA microarrays, various subtypes in breast cancer have been identified. The characterization of different subtypes under the breast cancer scope has allowed for an improved therapeutic approach. Specifically, the basal breast cancer (BBC) subtype is complex, with a poor prognosis (Bertucci et al., 2012). Glioblastoma multiforme (GBM) is the most prevalent type of tumor of the central nervous system. GBM has high metastatic abilities and an aggressive phenotype which poses a challenge on surgical removal giving patients an average 15 months of survival (Cheray et al., n.d.; Coleman et al., 2018) Due to BBC and GBMs complexity, researchers work to elucidate their intricacies to pave way for effective targeted therapies. Heterogeneity within individual tumors and across patients makes it difficult to identify individual signaling pathways that can be translated to a clinical setting (Coleman et al., 2018; Yoo et al., 2013).

Both BBC and GBM harbor stem cell signatures that are associated with self-renewal, metastasis, and chemotherapeutic resistance. Despite therapeutic advances, targeting the bulk of tumorigenic cells does not exclude patients from future metastasis and relapse (Murat et al., 2008; Sin & Lim, 2017; Yu, et al., 2012). Understanding pathways that drive the stem signatures in GBM and BBC would aide in the development of therapeutic drugs that decrease patient relapse.

The phosphatydilinositol-3 kinase (PI3K) pathway is of interest due to its upregulation in cancer stem cell populations (Xia & Xu, 2015). The PI3K pathway becomes activated through insulin or growth factor binding to the receptor tyrosine kinase (RTK). Active PI3K will convert phosphatidylinositol-4,5, bisphosphate (PIP_2) to phosphatidylinositol-3,4,5 trisphosphate (PIP_3). PIP_3 membrane recruits and activates AKT which phosphorylates FOXO transcription factors (Foxo-1, -3, and -4) among other substrates. This phosphorylation of FOXO factors maintains their cytoplasmic localization, preventing them from binding to target genes and inducing transcription within the nucleus (Fu & Tindall, 2010). FOXO transcription factors regulate metabolism, apoptosis, tumor suppression and cellular differentiation (Martins et al., 2016). Inactivation of FOXO transcription factors through the constitutive activation of PI3K pathway is seen in many contexts to enable tumor survival (Fu & Tindall, 2010). AKT is also one of the proteins within mammalian target of rapamycin (mTOR) pathway. mTOR complex 2 (mTORC2) activates AKT to inhibit FOXOs pro-apoptotic role. AKT also activates mTOR complex 1 (mTORC1) to promote pathogenic progression via increased translation (H. Li, Zeng, & Shen, 2014).

PTEN counteracts PI3K by performing the reverse enzymatic reaction in a normal cellular setting. Consequently, PTEN will lead to FOXO activation. PTEN is commonly mutated to an inactive form in BBC and GBM tumors due to its downstream effect as a tumor suppressor (J. Li et al., 1997). Theoretically, in a *PTEN-null* cell line with overactive PI3K, the FOXO factors should be inactive/cytoplasmic. Our lab found that despite constitutive PI3K pathway output, FOXO factors were localized, at least in part, in the nucleus and were regulating stem genes. Further work showed that stem signal transduction was impacted by FOXO transcription

factors in GBM cells at the receptor, signal transducers and activators of transcription 3 (STAT3), and gene expression levels.

STAT3 is active in tumorigenesis to contribute to proliferation, differentiation, and apoptosis. The JAK2/STAT3 pathway becomes constitutively active in cancer cells to accelerate cancer progression. STAT3 proteins become activated through Janus activated kinase (JAK) transducer upon cytokine binding. JAK2 will cross phosphorylate the STAT3 proteins which will homodimerize and then will regulate gene expression of pluripotency genes in the nucleus (Sansone & Bromberg, 2012).

This led us to investigate their interaction in the propagation of a stem cell signature and identify if the simultaneous inhibition of PI3K/mTOR and JAK2 would serve as a tumor therapeutic promoting apoptosis through the activation of FOXO transcription factors and decreasing the stem cell signature through STAT3 inhibition in these aggressive cancers. We treated with a PI3K-AKT-mTOR inhibitor drug (NVP BEZ235) as well with a JAK inhibitor (JAKi). This combination treatment effectively induced apoptosis but also led to an enrichment of the stem cell population.

CHAPTER II

LITERATURE REVIEW

FOXO Transcription Factors

The first Forkhead box class O (FOXO) transcription factor identified was FoxO1, originally labeled FKHR (Galili et al., 1993). FOXO1 was later characterized as a fusion protein to PAX3 promoting a strong transcriptional output for oncogenesis in Alveolar Rhabdomyosarcomas (Fredericks et al., 1995). Since then, four main related members have been found to be part of the mammalian family of FOXO factors; FOXO1-3a, -4, and -6. FOXO6 is the most distantly related factor in this family and is not regulated in the same way by AKT (Tzivion et al., 2011). The remaining FOXO proteins-1,-3a, and, -4 have become a target of interest due to their contributions to various crucial cellular processes such as; cell cycle arrest, inducers of apoptosis, oxidative-stress resistance, and as stem cell regulators (Martins et al., 2016). Canonically, FOXO factors are required for development in mammals, with overlapping functions as well as independent importance. A *FOXO1* knockout in mice led to improper vascularization, this degree of abnormalities caused embryonic death on day eleven of development (Furuyama et al., 2004). *FOXO1* overexpression in mice endothelial cells showed an increasement in cell size, and led to death due to heart failure (Dharaneeswaran et al., 2014). *FOXO3a* and *FOXO4* null mice did not show issues in vascular development. However, the *FOXO3a* female mutants had reproductive development issues that led to their infertility. In addition, *FOXO4* null mutant mice did not express a consistent phenotype (Hosaka et al., 2004)

PI3K-AKT-mTOR Pathway

The PI3K/AKT pathway is upregulated in various types of cancers and has become a target of inhibitory drug therapy (H. Li et al., 2014). FoxO-1, -3, and -4 contain AKT phosphorylation sites which allow AKT to negatively regulate their transcriptional roles. Phosphorylation by AKT provides a binding site for 14-3-3 proteins which consequently blocks the DNA binding domain of the FoxO-1, -3a, and -4 transcription factors (Tzivion et al., 2011). PTEN is a tumor suppressor that counteracts PI3K with its role as a phosphatase protein that convers PIP_3 back to PIP_2. The *PTEN* gene is one commonly mutated in glioblastomas, breast cancers and prostate cancers (J. Li et al., 1997). Canonically, FOXO phosphorylation by AKT activation promotes cell growth because it prevents FOXOs translocation into the nucleus to activate apoptotic genes such as the *Fas ligand,* encoding a ligand of a caspase cascade. Downregulating PI3K output as well as consequent AKT activation will promote FOXO activation and nuclear localization to promote FOXOs activation of death ligands; Fas, Bcl-2, Bim (Brunet et al., 1999; Fu & Tindall, 2010)

mTOR regulates growth and survival as two separate complexes mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 binds through the help of regulatory associated protein of mTOR (Raptor) and mLST8 (GbetaL) aids in the phosphorylation of the target. AKT inhibits a mTORC1 inhibitor; proline-rich AKT substrate 40 kDa (PRAS 40) (Saxton & Sabatini, 2018). AKT also inhibits tuberous sclerosis complex proteins (TSC2). Inactive TSC2 will no longer inhibit Ras homologue enriched in the brain (Rheb) and it will successfully activate mTORC1 (Menon & Manning, 2013). The activation of mTORC1 will activate p70S6K, leading to 4E-binding protein 1 (4E-BP1) phosphorylation to promote tumorigenic advancement through protein and nucleotide synthesis as well as inhibiting

autophagy. mTORC2 interacts with RICTOR for target binding and MLST8. The mTORC2 complex phosphorylates AKT on serine 473 thereby increasing catalytic activity (Saxton & Sabatini, 2018).Given that the PI3K pathway contains feedback mechanisms to ensure sustained activity, we chose to employ a dual- PI3K pathway inhibitor NVP-BEZ235 that targets both PI3K and mTOR simultaneously. With a dual third-generation PI3K/mTOR inhibitor both pathways can be halted (H. Li et al., 2014).

JAK2/STAT3 Pathway

JAK2 is associated with cytokine receptors such as GP130 that are activated through growth factors or cytokine binding (IL6, IL10, IL21, IL27, G-CSF, leptin and LIF). STAT3 proteins are recruited and phosphorylated on tyrosine 705 by JAK1 and JAK2, which activates them for transcription. Unlike the transient activation of STAT proteins in normal cells, STAT-1, -3, and -5 are constitutively active and nuclear in tumorigenic cells. Of the 3 upregulated STAT proteins, STAT3 is the one found most commonly activated in cancer. This hyperphosphorylation is due to increased cytokine production (autocrine and paracrine), increased receptor levels, increased STAT protein production, and decreased levels of negative regulators of STAT proteins (Sansone & Bromberg, 2012). Chronic inflammation induced the development of lung tumors due to increasement of STAT3 phosphorylation. When the immune response in the lungs is triggered, there is an upregulation of cytokines to induce inflammation. This cytokine overexpression in bi-transgenic mice increased the activation of STAT3 and the occurrence of bronchoalveolar adenocarcinoma (Y. Li et al., 2007).

Cancer Stem-Like Cells

Cancer stem-like cells (CSCs) are a dangerous subpopulation within a heterogenous tumor. These cells are capable of self-renewal and unresponsive to chemotherapy. They are difficult to target because they are quiescent, resistant to oxidative stress, efficient at fixing DNA damage, and quick at exporting cytotoxic agents. Since more than 90% of cancer deaths are caused by a metastatic tumor, it is imperative to gain a better understanding of CSCs mechanisms (Yoshida, 2017). CSC's state of quiescence in the G0 phase prevents treatment damage because the cells are not cycling. In a $CD44^{+}CD24^{-}$ stem cell-like breast cancer subtype the JAK2/STAT3 pathway was found constitutively active and a potential target of inhibitory treatment (Marotta et al., 2011). CSCs may arise from stem cells that become cancerous or transform from already differentiated cells. Breast cancer cells have been effectively induced to become CSCs through interleukin-6 (IL6) cytokine expression. IL6 cytokine induced a positive feedback loop that involved STAT3 and PTEN among other signaling pathways (Iliopoulos et al., 2011). In some settings FOXO3 regulates hematopoietic stem cell maintenance as well as neural stem cell homeostatic program (Miyamoto et al., 2007; Renault et al., 2009; Zhang et al., 2011). Known stem cell markers are often tracked to identify the enrichment of stemness within populations. These markers further contribute to the enhancement of their stem cell signature; SOX2, OCT4, Bmi1, and NANOG (Cheray et al., n.d.). Mechanisms that regulate the stem cell program are poorly understood. Successful identification of vital mechanisms through which CSCs are maintained will allow for the development of improved therapies against CSCs initiation and progression. There is abundant evidence that PI3K/mTOR/STAT3 are contributing to the stem cell signature. Elucidating the mechanisms within these complex pathways can help in the development of targeted therapies.

CHAPTER III

METHODOLOGY AND FINDINGS

Maintenance of Cell Lines

The glioblastoma cell line (U87MG) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown under standard conditions of 5% CO_2 at 37° C in Minimal Essential Media Eagle (MEM) supplemented with 10% Bovine Serum (FBS) and 5% penicillin/streptomycin anti-fungal anti-bacterial. The breast cancer cell line (BT549) was maintained under same conditions of U87MG with Roswell Park Memorial Institute (RPMI) as the base media used.

Experiment 1: Did the Drug Treatments Effectively Inhibit STAT3 and AKT Phosphorylation?

Pharmacologic Inhibition of STAT3: JAKi was purchased from Sigma, St. Louis, MO. JAKi treatment (2μM final concentration) will inhibit the activation of STAT3 protein through the inhibition of phosphorylation at Y705.

The nuclear localization of STAT3 is increased by tyrosine phosphorylation on Y705 by Janus kinase 1 (JAK1) (Darnell, 1997; Meyer & Vinkemeier, 2004). Inhibition with JAKi reduces STAT3 nuclear localization (Darnell, 1997; Murase & Mckay, 2014). Therefore, we employed JAKi (2μM final concentration) to pharmacologically inhibit STAT3 nuclear localization and tested whether this impacted cell growth alone or in combination with

NVP-BEZ235 treatment (50nM final concentration). We examined total STAT3 and phospho-STAT3 Y705.

Pharmacologic Inhibition of PI3K and mTOR: NVP-BEZ235 was purchased from Sigma (Saint Louis, MO), and utilized at a final concentration of 50 nM in indicated experiments. U87MG and BT549 cells were plated at a density of 2,700 cells per mL and were treated for 5 days with NVP-BEZ235.

Protein lysates were utilized to assess drug therapy effectiveness for inhibiting STAT3 (JAKi) and PI3K/mTOR (NVP-BEZ235). Specifically, AKT activation leads to the phosphorylation of FOXO transcription factors and cytoplasmic localization. Inhibition of PI3K and mTOR prevents the positive feedback loop preventing FOXOs from translocating to the nucleus and inducing apoptosis. Therefore, we employed a PI3K/mTOR dual inhibitor to pharmacologically inhibit AKT and sequentially induce FOXO nuclear activity. We examine total AKT and phospho-AKT with GAPDH as a loading control.

Western Blot

Expression of STAT3 and AKT activation were assessed by western blot analysis. Protein extracts were prepared from BT549 and U87MG cells by rinsing the 6-well plate with 1XPBS followed by directed 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 then added to each well and scrapped. The lysate was collected from the wells and transferred into 1.7 ml microcentrifuge tubes and heated for 10 minutes at 37°C in a dry bath heat block at 95°C. Equal amounts of protein lysates were then resolved by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) for 1 hour at 100 voltage. The protein was transferred onto a polyvinylidene fluoride (PVDF) membrane for 1 hour and 30

minutes at 125 voltage. The PVDF membrane was blocked in a 5% milk solution containing 1x Tris-buffered saline with Tween 20 (TBST) and Carnation non-fat dry milk for 1 hour. The blocking solution was rinsed off with 1 x TBST and the PVDF membrane was incubated overnight at 4°C with either of the following antibodies: P-STAT3 Y705, P-AKT S473, T-STAT3, T-AKT and GAPDH. After overnight incubation the membrane was rinsed for 20 minutes in 5 minute intervals with 1 x TBST then incubated in secondary antibody for 1 hour. After secondary incubation, membranes were washed again for 20 minutes in 5-minute intervals and developed using Supersignal West Dura Extended Duration Substrate luminol solution for 5 minutes. The BioRad Chem Doc XRS+ molecular imager was used to photograph the blot while exposed for protein identification.

Antibodies were obtained from Cell Signaling Technologies (Danvers, MA): total STAT3 (9139T), phosphor STAT3 tyrosine 705 (9145T), total AKT (C67E7), and Phospho AKT serine 473 (9271S). GAPDH (G-9) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX).

Experiment 1 Results:

The lysates collected from the JAKi drug treatment showed no P-STAT3 Y705 signaling but the T-STAT3 signal still appears. The lysates collected from the NVP-BEZ235 drug treatment greatly reduced P-AKT S473 but not the T-AKT signal. The GAPDH loading control confirms the same amount of protein was loaded across samples (Figure 1).

Experiment 2: Will the Double Treatment Affect Cell Growth More than Single Treatment?

Cell growth was tracked through crystal violet staining with each treatment and quantified for comparison with the control. The growth curves will show the effect of the treatments on the growth of both U87MG and BT549 cell lines.

Plate Collection

Cells were plated on twelve well plates with 15,000 cells per well and each treated with dimethyl sulfoxide (DMSO), JAKi, NVP-BEZ235, or a combination of both JAKi and NVP-BEZ235. Drug treatments were applied in triplicate and a plate for each GBM and BBC was collected every 24 hours for 5 days through crystal violet staining. Plates were aspirated of media then each well was washed with 0.5 ml of 1x phosphate buffered saline (PBS) once before being stained with 0.5 ml of crystal violet stain (0.5% crystal violet in buffered formalin) and incubated for 15 minutes. The stain was aspirated and wells were washed 3 times with 0.5 ml of 1x PBS.

Plate Quantification

After 5-day 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 590nm. Readouts of each plate were transferred to excel for analysis.

Experiment 2 Results:

Quantified plates were analyzed with a Tukey test on vassarstats.net for statistical significance. In the U87MG cell line on day 5, each treatment was found to have negatively affected cell growth from the DMSO vehicle ($P < .01$). The JAKi single treatment had a significantly lower growth inhibition ($P < .01$) when compared to single NVP-BEZ235 and double treatment of JAKi/NVP-BEZ. However, there was no significant difference between the single NVP-BEZ235 treatment and the JAKi/NVP-BEZ235 double treatment (Figure 2). In the

BT549 cell line on day 5, each treatment was found to have negatively affected growth from the DMSO vehicle ($P < .01$). However, there was no significant difference between JAKi, NVP-BEZ235, and JAKi/NVP-BEZ235 dual treatment (Figure 3). Error bars were added using the standard error of the collection readouts completed in triplicate.

Experiment 3: Will Apoptotic Gene Expression Increase with Double Treatment and Stem Cell Gene Expression Decrease?

Gene expression analyses allowed us to identify mechanisms driving the cells when treated with the combination therapy as well as with the individual drugs. Primarily *OCT4*, *SOX2* and *NANOG* regulated by STAT3 are a key component of the maintenance and promotion of stemness (Wang et al., 2018). FOXO has also been linked to *OCT4* and *SOX2* activation in certain settings such as stem cells but the mechanism was not fully understood (Zhang et al., 2011). Through the truncation of the stemness positive feedback loop promoted by JAK/STAT3 we aimed to decrease the stem signature and rerouting of FOXO to promote cell death. We tracked apoptotic genes FOXO is known to induce: *TRAIL*, *BIM*, and *FAS*. The dual treatment was intended to potentially cause an epigenetic shift to reroute FOXO and induce apoptosis while simultaneously decreasing the stem phenotype that drives these aggressive cancers.

Quantitative Real Time PCR

Total RNA was prepared using the Qiagen RNeasy kit (Hilden, Germany), which was then used to generate cDNA using Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). Samples (cDNAs) were analyzed using (Power SYBR Green Master Mix, Applied Biosystems, Foster City, CA) and Illumina Eco Real-time system (San Diego, CA). Expression levels were normalized to *GAPDH* in gene expression experiments. Primer sequences are detailed in supplemental Table 1.

Experiment 3 Results:

BT549 Apoptosis. *BIM* was only induced by the NVP-BEZ235 treatment ($P < .05$) (Figure 4).

BT549 Stemness. *OCT4* expression was robustly induced ($P < .01$) only by NVP-BEZ235 (Figure 7). *LIF* expression was induced by NVP-BEZ235 ($P < .01$) and the double treatment ($P < .01$) as well (Figure 9).

U87MG Apoptosis. *BIM* gene was analyzed and although it was induced significantly by each treatment ($P < .01$) it had a larger induction by the double treatment ($P < .05$) (Figure 5). *TRAIL* expression was induced by NVP-BEZ235 ($P < .01$) and double treatment ($P < .01$) (Figure 6).

U87MG Stemness. *OCT4* showed a 20fold induction by NVP-BEZ235 ($P < .01$) and a 25fold induction by the double treatment ($P < .01$) (Figure 8). *ALPL* was induced by every treatment ($P < .01$) with almost a 2fold induction by JAKi, a 3fold induction by NVP-BEZ235, and 6fold induction by the double treatment (Figure 10). *NANOG* was equally induced by NVP-BEZ235 ($P < .01$) and the double treatment ($P < .01$) (Figure 11). *SOX2* expression was induced by NVP-BEZ235 ($P < .01$) and the double treatment ($P < 05$) (Figure 12).

Experiment 4: Will Apoptosis be Induced Higher in the Double Treatment?

The drug treatment time course made it challenging to accurately track the apoptotic gene expression with qPCR so to acquire a precise readout, flow cytometry was performed. Marker for early apoptosis annexin V conjugated with green fluorescent dye FITC was used to bind to the externalization of phospholipid phosphatidylserine thereby detecting membrane depolarization. A DNA intercalating agent and late marker of apoptosis propidium iodide (PI) was used to detect cell permeability (Rieger, et al., 2011).

Flow Cytometry

Growth medium was aspirated after a 5-day treatment. Cellss were trypsinized with 2 ml of 0.25% trypsin each and incubated for 5 minutes at room temperature: control, double dose DMSO, JAKi, NVP-BEZ235 and, both JAKi and NVP-BEZ235. 1.5 ml of each plate was transferred into 1.7 ml microcentrifuge tubes and centrifuged at G 0.2 for 10 minutes. Trypsin supernatant was aspirated and 0.1 ml of 1x PBS was resuspended in each microcentrifuge tube to wash and then centrifuged at G 0.2 for 10 minutes. 1x PBS supernatant was aspirated and cells in microcentrifuge tubes were then resuspended with dilute binding buffer. 100 µl of each sample diluted with binding buffer were added to a fresh microcentrifuge tube. Each tube was then added 2 µl of annexin V FITC (Mitochondrial Membrane Potential/Annexin V Apoptosis Kit) and 1 µl of PI and incubated for 15 minutes in the dark. 400 µl of binding buffer was added after the 15-minute incubation and cells were sorted by BD FACSCelesta flow cytometer (BD Biosciences, San Jose, CA).

Experiment 4 Results:

There was a statistical increase ($P < .01$) of annexin V FITC and propidium iodide in each of the treatments when compared to the DMSO control group. The double treatment apoptotic induction had no difference from the induction of apoptosis by JAKi (Figure 13).

CHAPTER IV

SUMMARY AND CONCLUSION

The sole use of PI3K inhibitors have previously shown to only halt growth. Through this time period in which cells are not growing they could develop resistance to treatments and potentially become metastatic (Zwang et al., 2017). In these specific cell types, the stem cell subpopulation poses a bigger challenge because of their quiescent state (Yoshida, 2017). It is therefore imperative to identify treatments that will induce apoptosis and decrease the stemness within tumors. AKT S473 and STAT3 Y705 expression was assessed through western blot analyses and their signal was effectively decreased in the treated samples. Total AKT and total STAT3 expression was assessed along with GAPDH as a loading control. Crystal violet staining allowed us to initially quantify the growth of cells under the single and double treatment. There was no significant difference between NVP-BEZ235 and the double treatment. In order to delineate more accurately the induction of apoptosis, flow cytometric analyses were performed. Surprisingly the induction of apoptosis significantly increased in each treatment when compared to the control ($P < .01$). The induction was higher in the single JAKi and the double JAKi/NVP-BEZ235 treatments with no difference noted between them. We also evaluated expression of apoptotic genes: *TRAIL* and *BIM*. PI3K inhibition with NVP-BEZ235 did not clearly lead to the fold induction of known FOXO factor apoptotic genes. Apoptotic expression was difficult to track due to the treatment cells being much less confluent than the DMSO vehicle group. Due to time constraints this issue was not fully addressed, but future analyses involves a time course

through which genes expression can accurately depict the apoptotic induction early on the treatment.

We hypothesized apoptosis would be induced by FOXO transcription factors in the double treatment. However induction and/or enrichment of a stem cell pluripotency signature was surprisingly increased. Through PI3K inhibition, we promoted FOXO nuclearization and found a robust increase in stem cell genes and markers. In the single NVP-BEZ235 and double treatment *OCT4*, *SOX2*, *LIF*, *NANOG*, and *ALPL* were strongly and significantly induced.

We found that in this setting, the FOXO transcription factors are driving the stem cell signature instead of the desired apoptosis. While there was little evidence of apoptosis we found abundant confirmation that the stem program was activated. This ease of stem program detection is because it is maintained, whereas the induced apoptosis signal would dissipate and become difficult to track with such a small degree of cell growth.

The majority of the cells underwent apoptosis with the NVP-BEZ235 and JAKi double treatment, but the remaining cells seem to have stemness enrichment. Implications on chemotherapy arise in which this stem cell niche enrichment or expansion would lead to a more aggressive tumor growth post therapy. Stem cells may also have an epigenetic landscape, which would prevent the easy induction of cell death. Further studies would be to determine if the remaining cells are quiescent and how to change their G0 stage to allow for targeted therapy.

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APPENDIX

TABLES AND FIGURES

Gene	Primer Sequences
GAPDH	F: GAAGGTGAAGGTCGGAGT R: GAAGATGGTGATGGGATTTC
OCT4	F: CTTGGGCTCGAGAAGGATGT R: CTGAGAAAGGAGACCCAGCA
SOX2	F: ACTTTTGTCGGAGACGGAGA R: CATGAGCGTCTTGGTTTTCC
NANOG	F: AGATGCCTCACACGGAGACT R: TTTGCGACACTCTTCTCTGC
ALPL	F: TGCCAGAGAAAGAGAAAGACC R: CTCCCAGGAACATGATGACA
LIF	F: CTATGTGGCCCCAACGTGA R: TTGAGGATCTTCTGGTCCCG
IL6	F: CCACTCACCTCTTCAGAACG R: AGGTTCAGGTTGTTTTCTGCC
TRAIL	F: CCAGAGGAAGAAGCAACACA R: GAATAGATGTAGTAAAACCCTTTTTCA
FAS	F: GGGGTGGCTTTGTCTTCTTCTTTTG R: ACCTTGGTTTTCCTTTCTGTGCTTTCT
TUBB3	F: GGCCTTTGGACATCTCTTCA R: ACCACATCCAGGACCGAAT
BIM	F: CAAACCCCAAGTCCTCCTT R: TCTTGGGCGATCCATATCTC

Table 1. Primers utilized for qRT-PCR

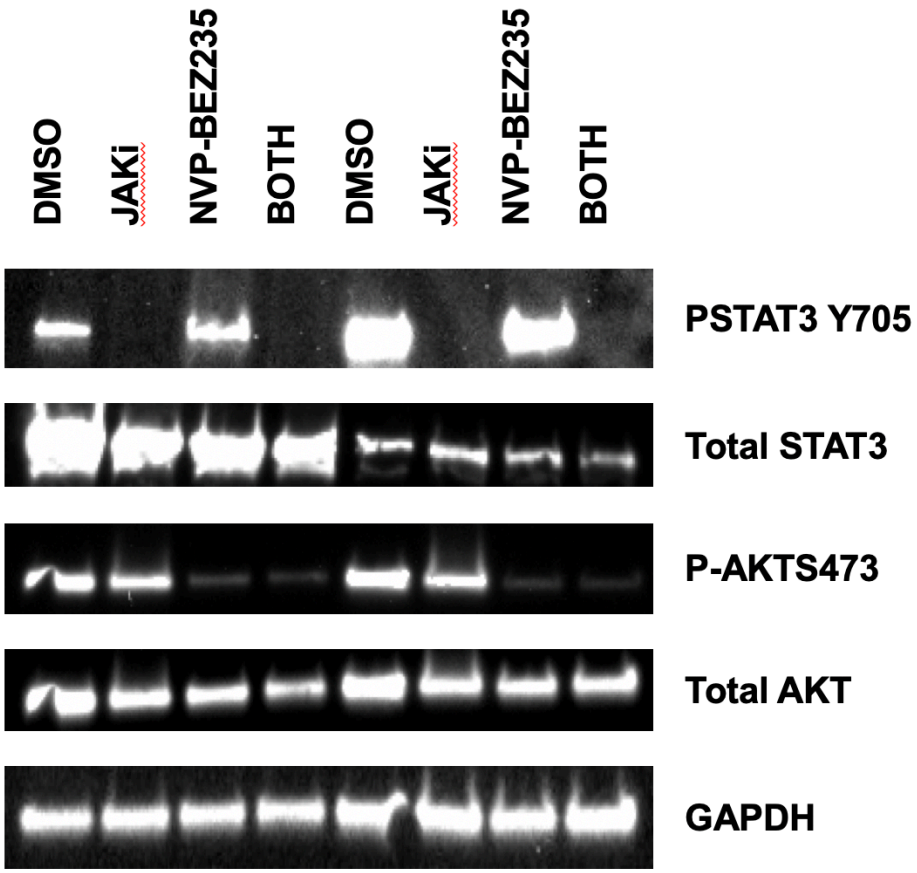

Figure 1: Western Blot Analysis of Phospho-AKT and Phospho-STAT3

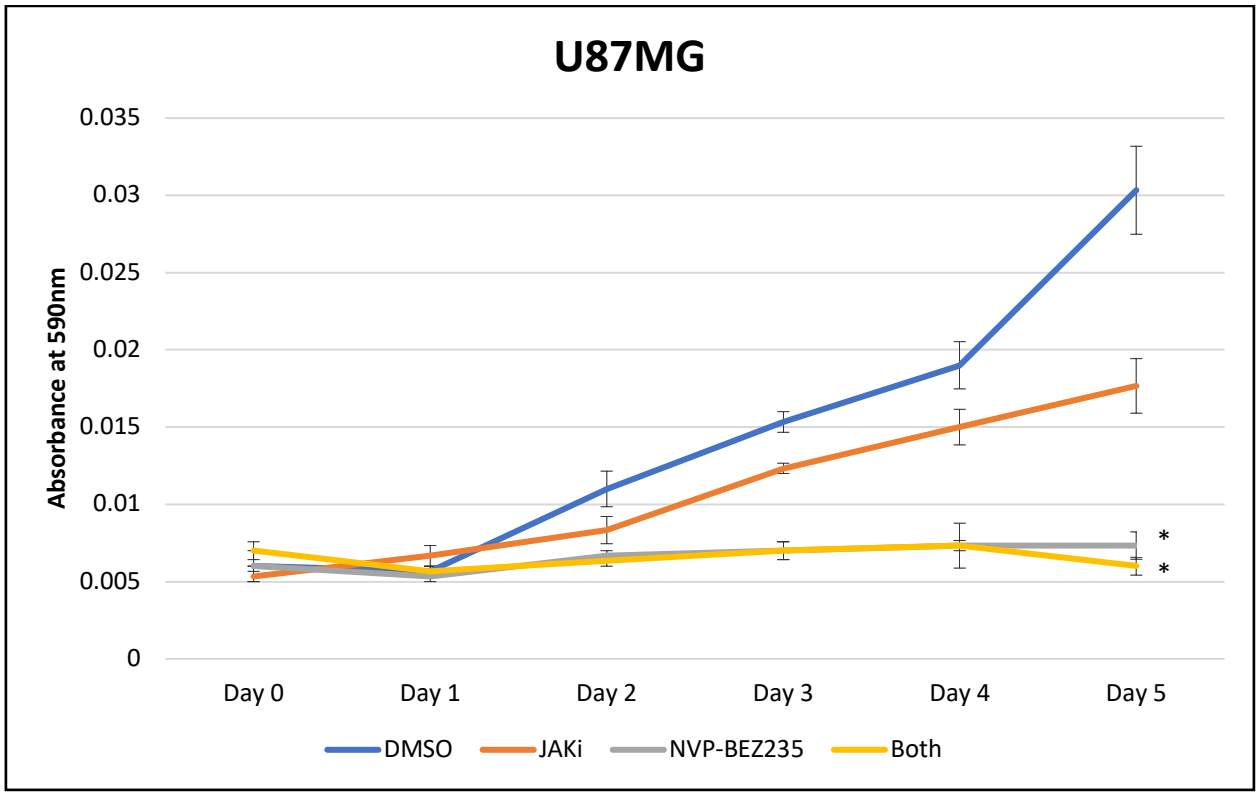

Figure 2: U87MG Growth Curves on 5-day Treatments. Tukey's test shows statistical significance from the control with SE bars.

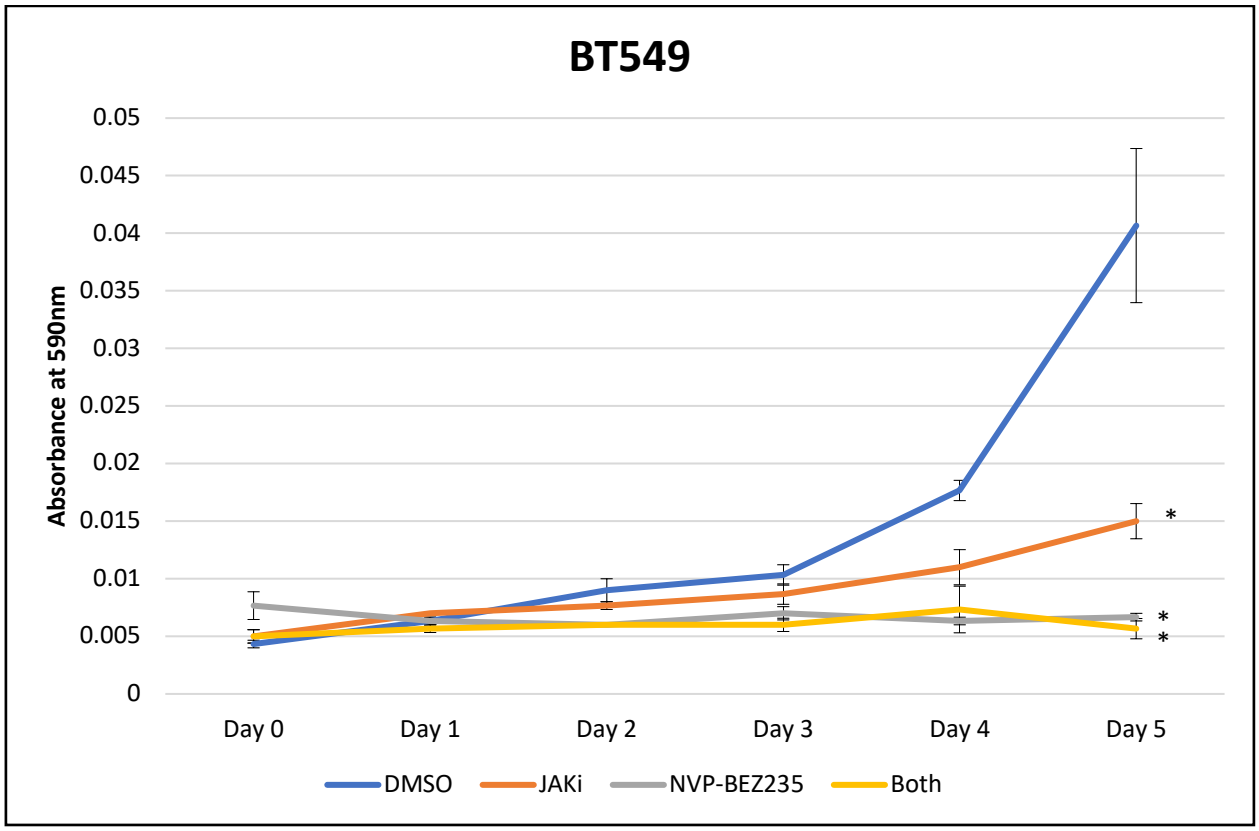

Figure 3: BT549 Growth Curves on 5-day Treatments. Tukey's test shows statistical significance from the control with SE bars..

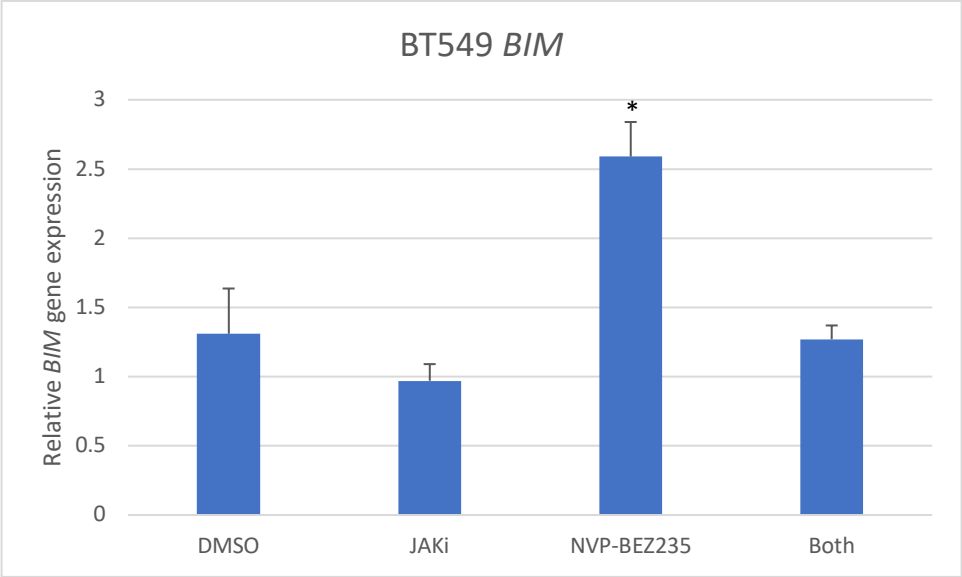

Figure 4: Apoptosis: Relative *BIM* Gene Expression in BT549. Student t-test shows statistical significance from the control with SE bars.

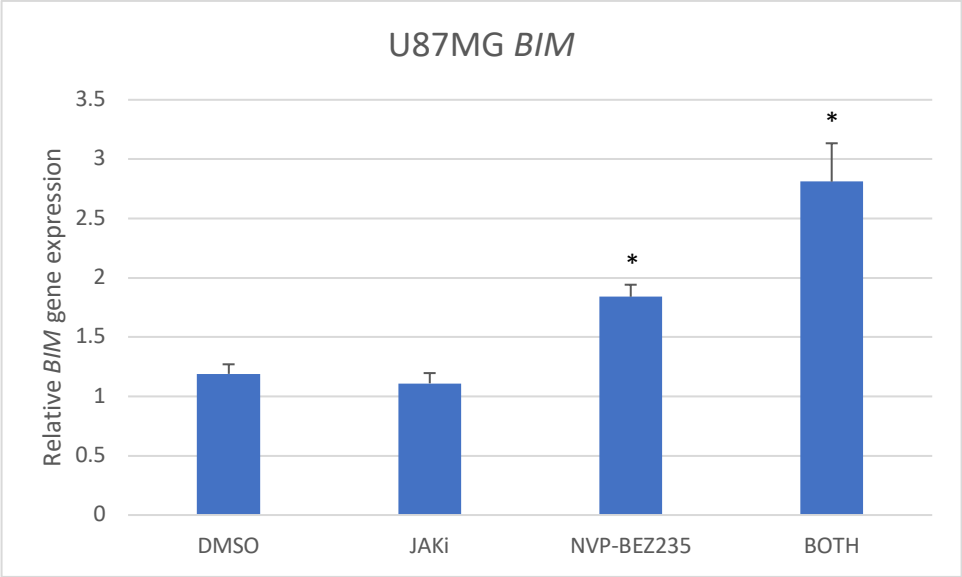

Figure 5: Apoptosis: Relative *BIM* Gene Expression in U87MG. Student t-test shows statistical significance from the control with SE bars.

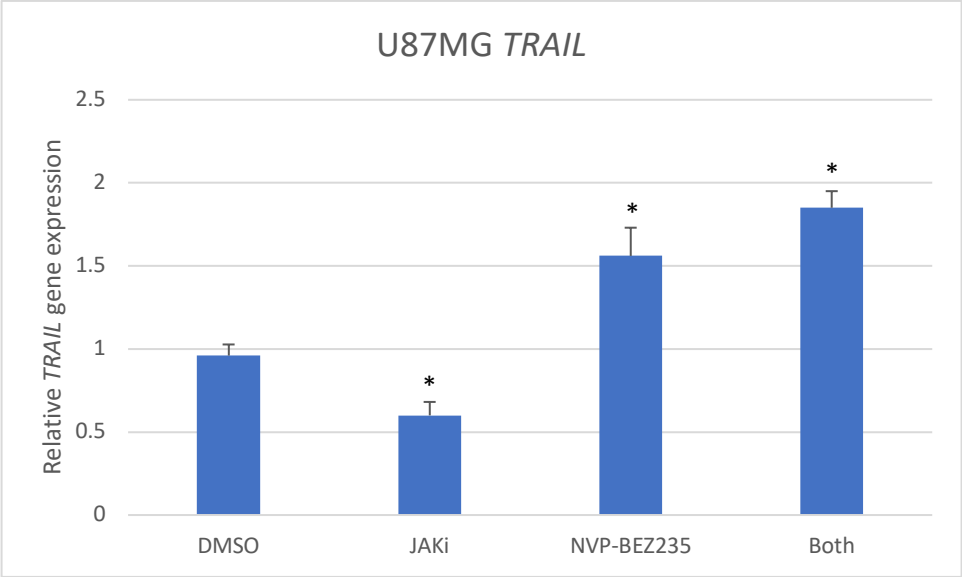

Figure 6: Apoptosis: Relative *TRAIL* Gene Expression in U87MG. Student t-test shows statistical significance from the control with SE bars.

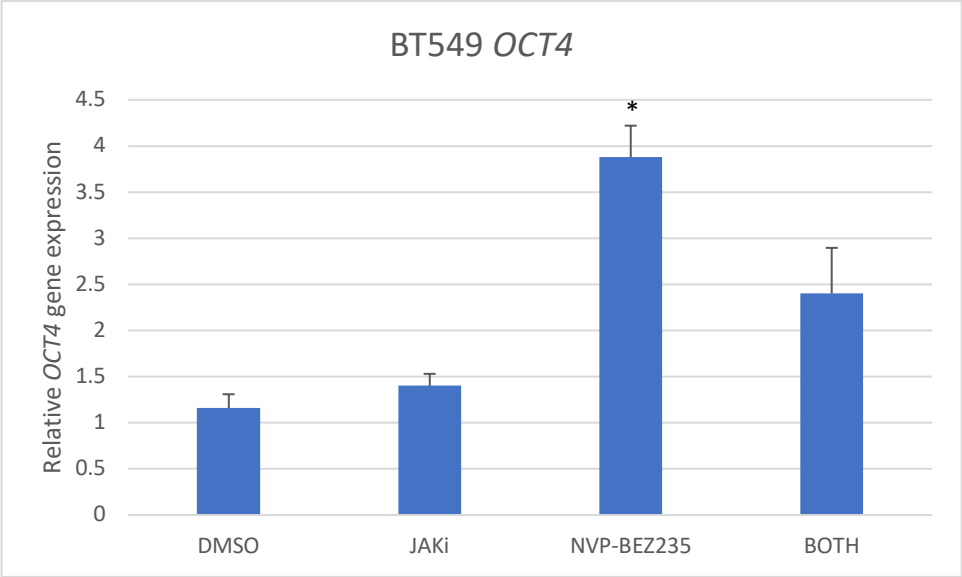

Figure 7: Stemness: Relative *OCT4* Gene Expression in BT549. Student t-test shows statistical significance from the control with SE bars.

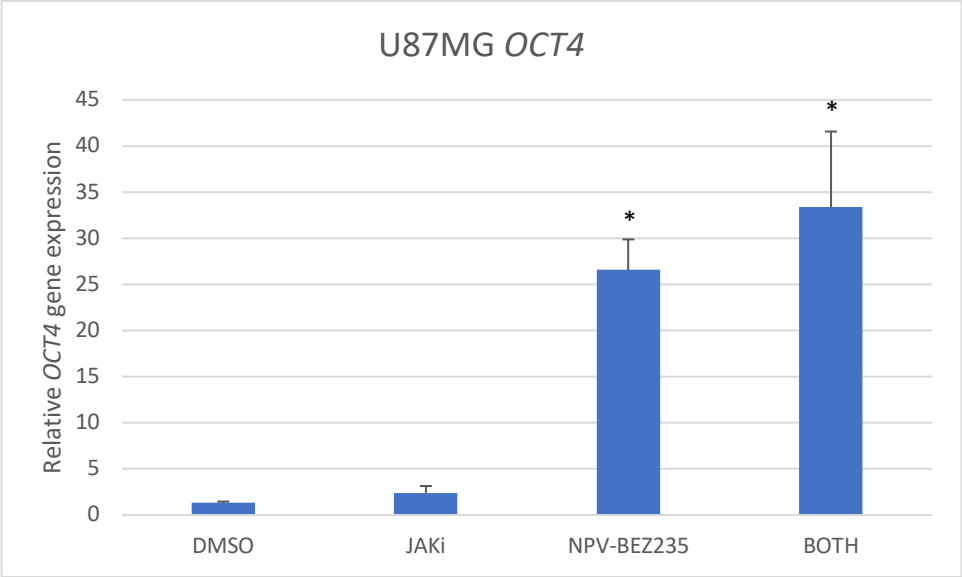

Figure 8: Stemness: Relative *OCT4* Gene Expression in U87MG. Student t-test shows statistical significance from the control with SE bars.

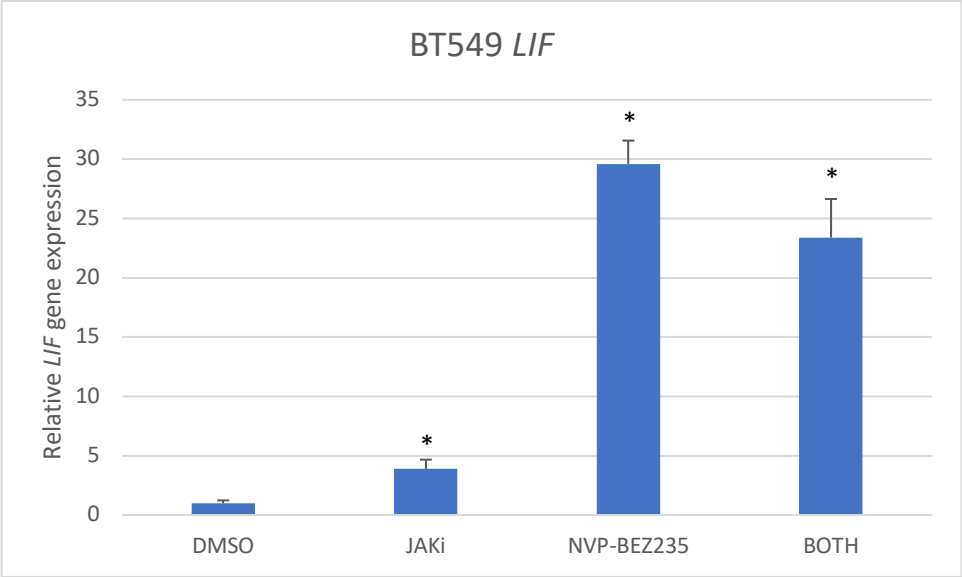

Figure 9: Stemness: Relative LIF Gene Expression in BT549. Student t-test shows statistical significance from the control with SE bars.

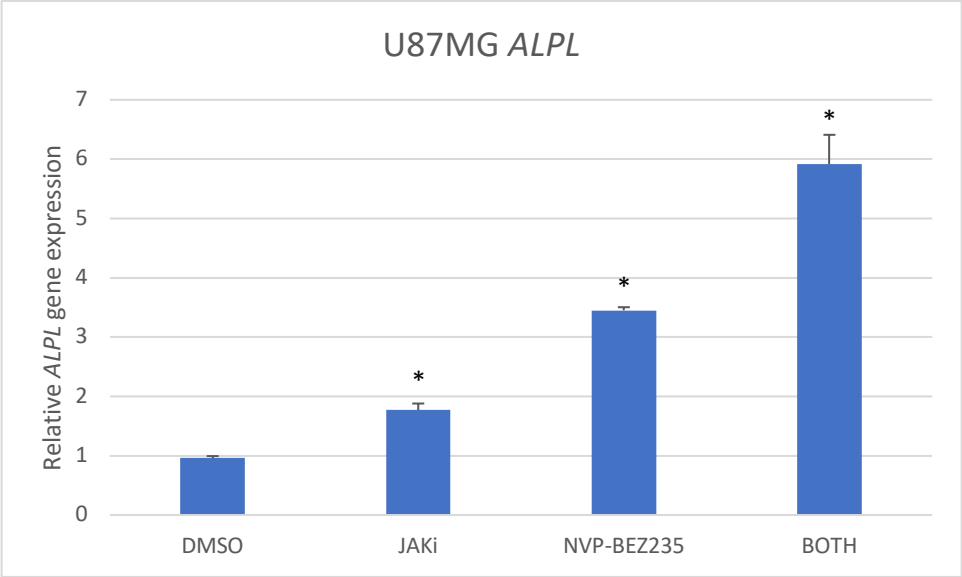

Figure 10: Stemness: Relative *ALPL* Gene Expression in U87MG. Student t-test shows statistical significance from the control with SE bars.

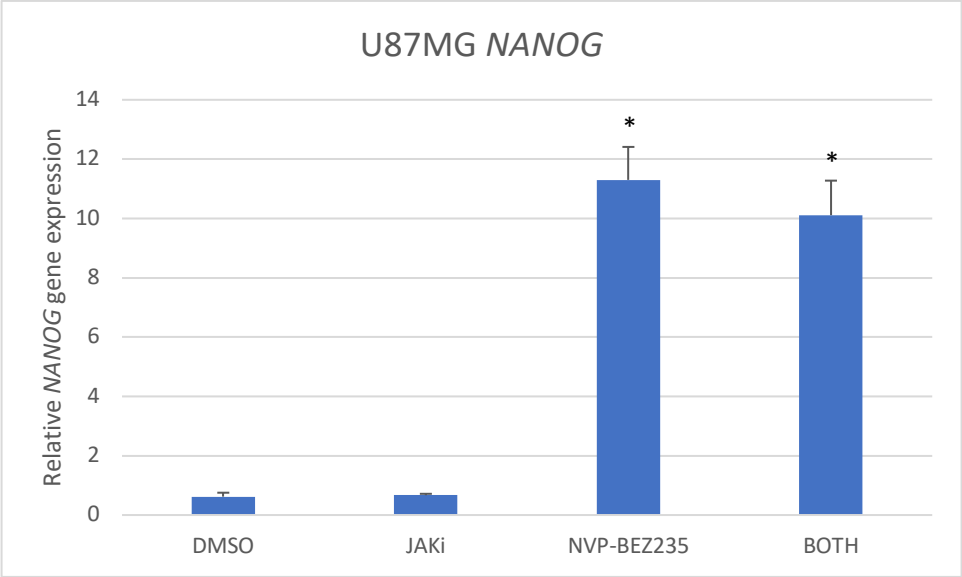

Figure 11: Stemness: Relative *NANOG* Gene Expression in U87MG. Student t-test shows statistical significance from the control with SE bars.

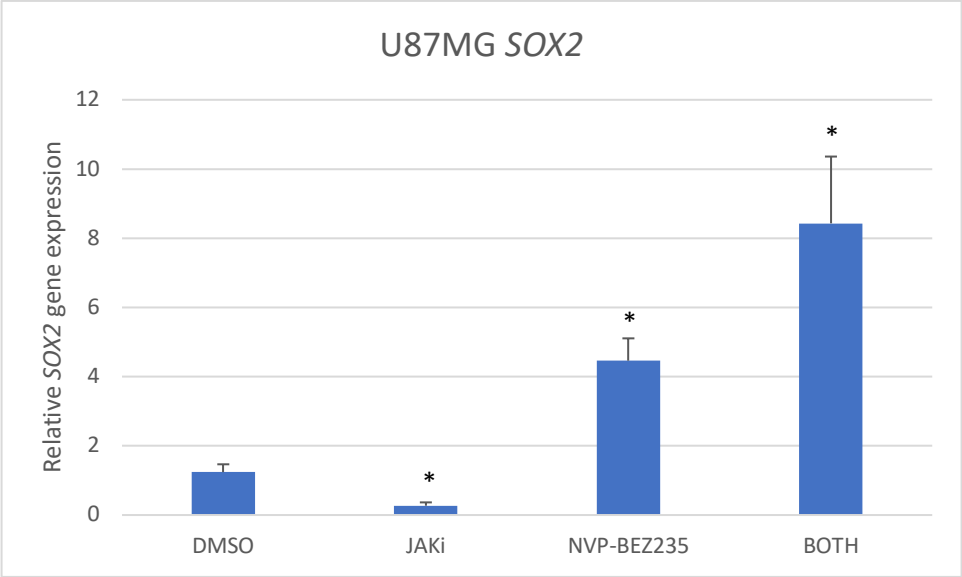

Figure 12: Stemness: Relative *SOX2* Gene Expression in U87MG. Student t-test shows statistical significance from the control with SE bars.

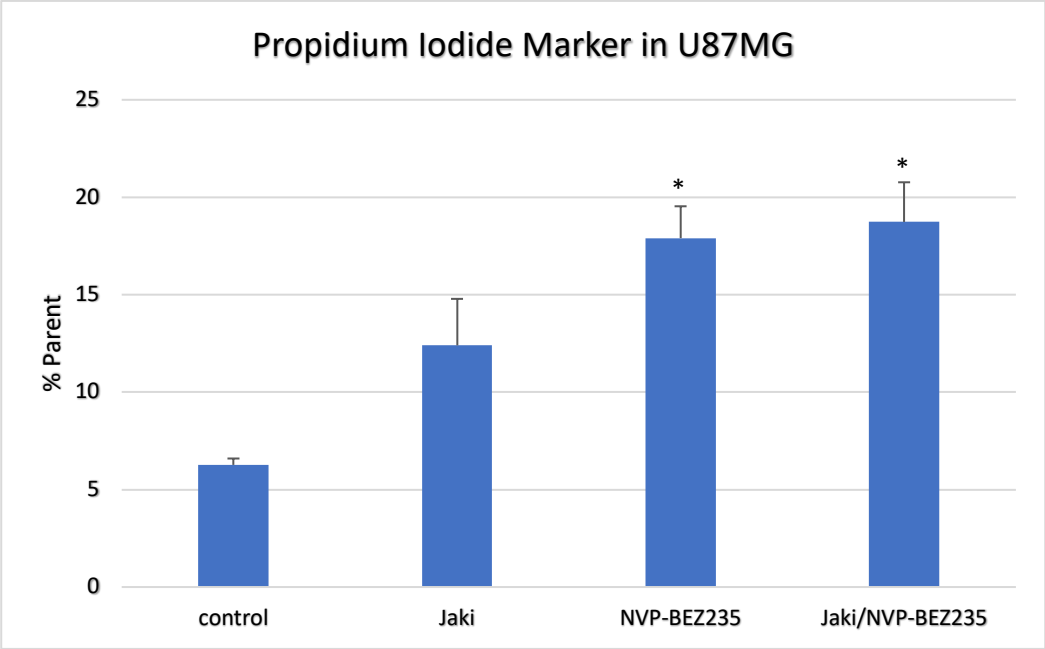

Figure 13: Flow Cytometry: Analysis of Late Apoptosis in U87MG Cell Line. Tukey's test shows statistical significance from the control with SE bars.

BIOGRAPHICAL SKETCH

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