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Effects Of Penfluridol On Integrin-Fak Signaling And Tumor Cell Killing In Combination With Oncolytic Hsv In Glioblastoma

Mitra Nair

EFFECTS OF PENFLURIDOL ON INTEGRIN-FAK SIGNALING AND TUMOR CELL KILLING IN COMBINATION WITH ONCOLYTIC HSV IN GLIOBLASTOMA

by

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By

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DEDICATION

To my maternal grandparents who bravely fought this terrible disease we call cancer.

I miss you every day.

Acknowledgements

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EFFECTS OF PENFLURIDOL ON INTEGRIN-FAK SIGNALING AND TUMOR CELL KILLING IN COMBINATION WITH ONCOLYTIC HSV IN GLIOBLASTOMA

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Abstract

Integrins are known to play an important role in activating multiple intracellular pathways, one of which is focal adhesion kinase (FAK). Phosphorylation of FAK can lead to the activation of various downstream signaling pathways that can increase tumor cell growth and proliferation, making it an ideal target for cancer therapeutics. Due to the fact that many FAK inhibitors are limited in their penetration of the blood brain barrier, we investigated the use of Penfluridol, an antipsychotic drug known to attenuate integrin expression at a transcriptional level, in combination with oncolytic herpes simplex I virus (oHSV) in a glioblastoma model. We examined the effects of oHSV on FAK signaling by looking at FAK phosphorylation at different time points post-infection and saw that FAK was activated by viral infection at earlier time points. When combining Penfluridol with oHSV, we saw that at higher drug concentrations there was a decrease in viral propagation, however combination treatment increased overall tumor cell killing compared to either treatment alone. Additionally, we know that viral treatment causes immune cell infiltration leading to viral clearance. Combination of Penfluridol with virus treatment significantly reduced immune cell migration towards infected cells. To assess the effects of Penfluridol in vivo, GBM tumors were implanted intracranially in mice and treated with Penfluridol and oHSV. Survival data and bioluminescence imaging to evaluate virus replication was collected to examine the efficacy of the combination treatment. Overall, virus replication was not inhibited by Penfluridol treatment *in vivo*. Furthermore, mice treated with both Penfluridol and oHSV survived significantly longer than mice in individual treatment groups. These findings conclude that Penfluridol has the ability to attenuate integrin and FAK signaling and increase tumor cell killing when combined with oHSV *in vitro*. Additionally, we have seen that the combination treatment of Penfluridol and oHSV can significantly enhance survival in both immune deficient and immune competent mouse models *in vivo*.

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Introduction

Glioblastoma (GBM) is currently the most commonly diagnosed primary, malignant brain tumor. The invasive nature of this tumor makes complete surgical resection with clear margins extremely difficult to accomplish without negatively affecting normal brain function (1). In addition to its invasive nature, GBM also secretes various cytokines and growth factors that encourage the infiltration of tumor supportive immune cells. This ultimately leads to the creation of a tumor microenvironment (TME) that influences tumor growth and metastasis, along with the ability for the tumor to resist treatment (2). Despite efforts that have been made towards developing treatments, the overall prognosis remains poor, making it imperative that other therapeutic approaches are investigated in order to improve the survival outcome in GBM patients (3). Oncolytic viral (OV) therapy is an approach that involves the use of either a natural strain or genetically engineered virus that can enter and replicate specifically in cancer cells. This will ultimately lead to the lytic destruction of cancer cells causing minimal damage to surrounding tissues (4). Currently, one of the most studied OV treatments is the use of the herpes simplex-1 virus (HSV) and the oHSV therapy, Talimogene laherparepvec (T-Vec), which is currently FDA approved for the treatment of metastatic melanoma (5). Additionally, many other second generation oHSV vectors are at various stages in clinical trials for GBM treatment (NCT00028158, NCT02062827, NCT00157703). Despite the numerous advantages of oHSV therapy, virally induced activation of pathways that can allow for viral clearance and tumor cell growth can pose an issue for the clinical development and application of viral therapy. Therefore, it is pertinent that we examine the mechanism behind these activated pathways and consider using oHSV in combination with another therapy to enhance overall tumor cell death. In this paper, we will explore the role that oHSV plays in activating integrins and in turn, activating

FAK which can ultimately allow for tumor cell growth/proliferation and viral clearance. Integrins are a family of cell surface receptors that bind extracellular matrix (ECM) proteins and activate numerous intracellular signaling pathways that are known regulate tumor cell proliferation and survival (6)**.** In addition to their signaling ability, integrins also play a role in viral entry. The gH/gL complex on the viron envelope interacts with high affinity to integrin receptors αvβ6 and αvβ8, which then allow the virus to enter the cell via plasma membrane fusion or endocytosis (7). One of the major pathways that is activated as a result of integrin engagement is focal adhesion kinase (FAK). FAK is known to regulate many aspects of metastasis and is overexpressed and activated in a variety of cancers. Furthermore, activation of FAK can lead to adhesion, migration, and invasion of tumor cells (8). Due to these downstream effects of integrin activation, we investigated the use of Penfluridol, an FDA approved antipsychotic drug, which has been shown to reduce integrin expression at a transcriptional level and decrease tumor cell growth in breast cancer tumor models (9). We hypothesize that combining oHSV with Penfluridol will decrease downstream activation of FAK, therefore, decreasing viral clearance, tumor cell survival, and tumor growth in our glioma model (Fig.1).

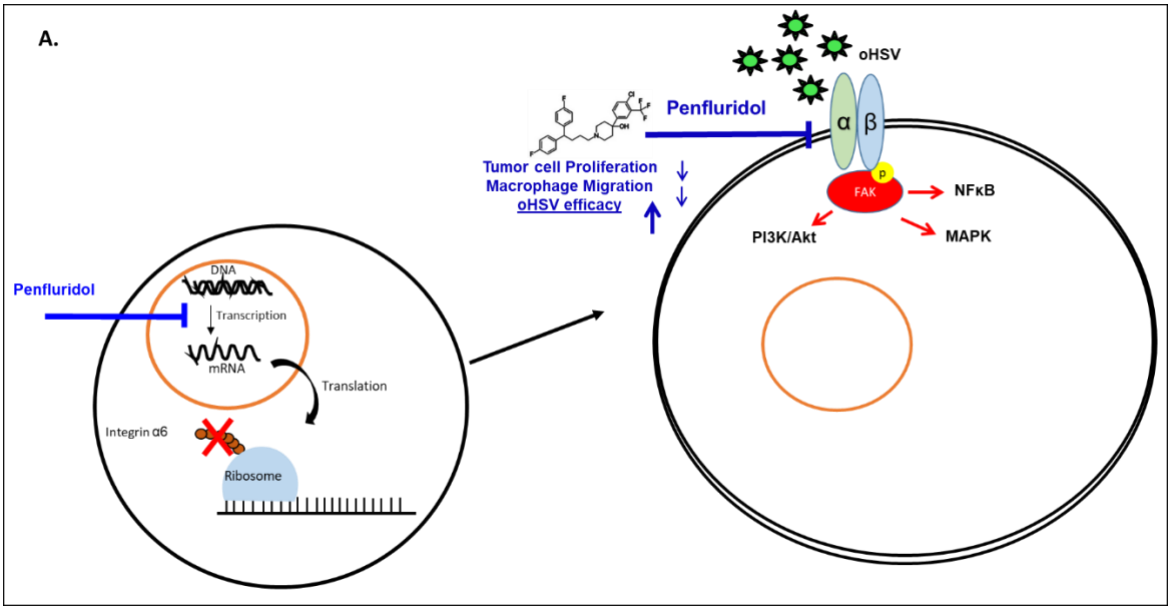

Fig.1 Graphical representation of hypothesis which illustrates the ability for Penfluridol (PF) to attenuate integrin and FAK signaling which can ultimately lead to a decrease in tumor growth and viral clearance.

Materials and Methods

Cell lines and Oncolytic Herpes Simples Virus-1 (oHSV-1)

U251T3, U87ΔEGFR, and Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gendepot, Houston, TX) supplemented with 10% fetal bovine serum (FBS). U251 cells were obtained from Dr. Erwin G. Van Meir (Emory University, Atlanta, GA) and U251-T3 (T3) cells were created in our laboratory (May 2009) as a tumorigenic clone of U251 cells by serially passaging these cells three times in mice. U87ΔEGFR cells express a truncated, constitutively active, mutant form of the epidermal growth factor receptor (EGFRvIII). Patient-derived primary GBM cells (GBM8, GBM12, GBM28, GBM43, and GBM1016) were provided by Dr. Jann N. Sarkaria (Mayo Clinic, Rochester, MN). Primary GBM cells were maintained as tumor spheres in neurobasal medium supplemented with 2% B27 without vitamin A, human EGF (20 ng/mL), and basic FGF (20 ng/mL) in low-attachment cell culture flasks. GL261N4 cells and monkey kidney epithelial derived Vero cells were obtained from the ATCC. Primary GBM cells (February 2019) were authenticated by MD Anderson Cancer Center Characterized Cell Line Core via STRS profiling and U87ΔEGFR (October 2018), and U251T3 (January 2015) cells were authenticated by the University of Arizona Genetics Core via STRS profiling. Murine BV2 microglia were maintained in DMEM supplemented with 2% FBS (2F). BV2 cells were obtained in January 2009 from J. Godbout (Ohio State University, Columbus, OH). Murine RAW264.7 macrophages were maintained in RPMI supplemented with 10% FBS. RAW264.7 macrophages were received in June 2010 from S. Tridandapani (Ohio State University, Columbus, OH). Murine BV2 and RAW264.7 cells have not been authenticated since receipt. All cells were maintained at 37 °C in a humidified atmosphere with 5% carbon dioxide and with 100 units of penicillin/mL and 0.1 mg of streptomycin/mL (Pen/Strep). All cells were routinely

monitored for changes in morphology and growth rate. All cells were confirmed negative for *Mycoplasma*. For oncolytic HSV, we used rHSVQ and rHSVQ-IE4/5-Luciferase (rHSVQ-Luc), which are both disrupted in the UL39 locus and deleted for both copies of the ICP34.5 gene. Viral stocks were generated and titered on Vero cells via standard plaque forming unit assays.

Western blot analysis and antibodies

Cell lysates were fractionated by SDS-PAGE and transferred to nitrocellulose (NC) membranes. Blocked membranes were then incubated with antibodies against Anti-integrin alpha V, Anti-integrin alpha 6, Anti-integrin beta 3, Anti-phospho Akt (S473), Anti-phospho p44/p42 MAPK (Cell Signaling Technology, Waltham, MA), Anti-phospho FAK (Abcam, Cambridge, MA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) (each diluted 1:1000) ; HRP-conjugated secondary anti-mouse antibody (each diluted 1:1000) (GE Healthcare, Piscataway, NJ); HRP-conjugated secondary goat anti-rabbit antibody (each diluted 1:1000) (Dako, Hamburg, Germany), and the immunoreactive bands were visualized using enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway, NJ).

Cell Proliferation Assay

Cells were plated in 96-well plates containing 10000 cells/well in 50uL of 2%FBS-containing media. Seeded cells were infected at 0.01, 0.05, and 0.1MOI with HSVQ in a volume of 25uL of 2%FBS-containing media and one hour later were treated with 4 different concentrations of either GSK2256098 or Penfluridol. Cells and virus were incubated for 24-72hpi at 37C, and cell viability was measured using a cell proliferation kit (Roche) following manufacturer’s protocol.

Absorbance was measured using a Synergy HT Multi-Mode Microplate reader (Biotek), and cell viability was measured as a percentage of uninfected control.

Quantitative virus spread by Cytation 5 live imaging

Glioma cells and primary GBM cells were infected with 0.5 MOI of HSVQ. One hour post virus infection, unbound virus was removed, and cells were treated with Penfluridol at 0.078uM, 0.156uM, 0.3125uM, 0.625uM and 1.25uM for 48 hours. Virus-encoded GFP expression was monitored every 4 hours for 48 hours using the Cytation 5 live plate reader/imager (BioTek Instruments). Captured images were analyzed by BioTek Gen5 version 2.06 software program. Prism (GraphPad) was used for curve fitting.

Cell Migration Assay

Cell migration was evaluated in a 24-well chemotaxis chamber equipped with a polycarbonate filter with 5 μm pores (Costar, Corning, NY). GBM8 tumor cells were plated in the bottom chamber and infected for 1 hour at a MOI of 1 and unbound viruses were washed out and treated with and without Penfluridol (5uM) for 2 hours. Subsequently, serum starved Raw264.7 or BV2 cells were plated in the top chamber and then left to migrate for 6 hours. Following incubation, cells were fixed and stained.

ELISA

Raw264.7 and BV2 cells were seeded in 6 well- plates and treated with 5uM of Penfluridol. All assays were performed in triplicate. Cells and media were harvested 24 hours post drug treatment, centrifuged for 5 minutes at 8,000 rpm, and cell pellets and supernatant conditioned media were frozen. Murine TNFα was quantified in cell supernatants using the mouse murine TNFα DuoSet ELISA kit (R&D Systems, Cat# DY410, Minneapolis, MN) according to the

manufacturer's recommendations. Serial dilutions of a known concentration of purified recombinant murine TNFα were used to establish a standard curve.

Xenograft studies

All mouse housing and experiments were performed in accordance with the Subcommittee on Research Animal Care of the Ohio State University (OSU) and the Animal Welfare Committee at the University of Texas Health Science Center in Houston guidelines and have been approved by the Institutional Review Board. Six-week-old outbred female athymic nu/nu mice were obtained from the Target Validation Shared Resource at the Ohio State University, from which original breeders (strain #553 and #554) were received from the NCI Frederick facility. NSG and C57BL/6 mice were obtained from Jackson Laboratory.

Three intracranial tumor studies were done in this paper. For intracranial tumor implantation, anesthetized mice were fixed in a stereotactic apparatus, and a burr hole was drilled at 2 mm lateral and 1 mm front to the bregma, to a depth of 3.5 mm. 1x10^5 GBM30 human glioma cells, 1x10^5 GBM12 human glioma cells, and 1x10^5 GL261N4 murine glioma cells were implanted intracranially. Ten days post tumor cell implantation, mice were randomized and injected with PBS or 1x10^5 pfu of rHSVQ1-Luc intratumorally. One day before PBS/oHSV injection, GBM12 and GL261N4 implanted mice began daily treatment of DMSO or Penfluridol (10 mg/kg) by oral gavage, while GBM30 implanted mice began daily treatment of DMSO or Penfluridol (10 mg/kg) by oral gavage after virus injection. Animals were observed daily and euthanized at the indicated time points or when they showed signs of morbidity (hunched posture and weight loss).

In vivo luciferase imaging

Nude mice bearing GBM30 tumors or NSG mice bearing GBM12 tumors were treated with DMSO or Penfluridol (10 mg/kg) via oral gavage daily starting at day 9 post tumor implantation. Mice were then treated with PBS or HSVQ1-Luc of 1x10^5 pfu by intratumoral injection 10 days post tumor implantation. Mice were subjected to *in vivo* luciferase imaging on days 1, 2, and 3, after the oHSV therapy. Mice were injected with Luciferin solution (25 mg/ml in PBS, dose of 100 mg/kg, Perkin Elmer, Waltham, MA) by an intraperitoneal injection and anesthetized. The anesthetized mice were placed on non-fluorescent black paper on the imaging platform under ZFOV-24 zoom lens-installed IVIS Lumina Series III Pre-clinical in vivo Imaging System (Perkin Elmer, Waltham, MA) to reduce background noise. Luminescence intensity was expressed as Averaged Radiance [p/s/cm²/sr], then normalized by tumor volume (mm^3).

Statistical analysis

To compare two independent treatments for continuous endpoints such as viral titers, and cell viability assay, student's t test was used. When multiple pairwise comparisons were assessed, one-way ANOVA was used. To evaluate the synergistic interaction between Penfluridol and oHSV, an interaction contrast or two-way ANOVA model was applied. Log-rank test was used to compare survival curves for survival data and Cox regression model was used to evaluate the interaction between Penfluridol and oHSV on survival data. P values were adjusted for multiple comparisons by Holms' procedure. A p value of 0.05 or less was considered statistically significant.

Results

Early activation of pFAK is consistent with engagement of virus with integrin receptors as a form of viral entry

To evaluate the role that the virus plays on FAK activation, we evaluated changes in cellular FAK phosphorylation upon treatment with oHSVQ. Patient derived primary GBM neurosphere cells (GBM12, GBM28, and GBM43) were plated on 6 well plates. Cells were treated with the indicated OV at an MOI (multiplicity of infection) of 1 for an hour, after which time unbound virus was washed off and cells were overlaid with media. Cell lysates from infected and uninfected cells were collected 1 and 8 hours post infection and probed for phosphorylated FAK by western blot analysis. Increased phosphorylation of FAK was observed early after infection in both the cells which was then reduced by 8 hours post infection. AKT is a central kinase that is downstream of FAK activation. To analyze changes in AKT pathway we probed for changes in AKT phosphorylation in these samples. Consistent with early FAK activation, we observed AKT phosphorylation as soon as an hour after infection. While FAK activation was lost at later time points, AKT phosphorylation was sustained even at later times after infection suggesting that FAK activation is temporal (Fig. 2).

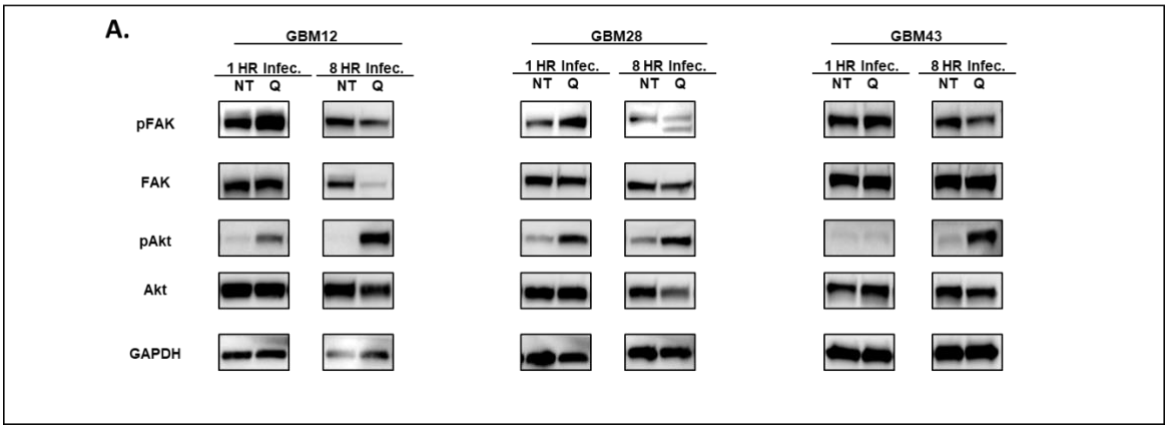

Fig.2 Effects of oHSV on FAK activation/signaling
GBM12, GBM28, and GBM43 were treated with 1 MOI (multiplicity of infection) of HSVQ and Enve virus. Cell lysate was collected and processed for western blot at two different timepoints post infection. Expression levels of pFAK and pAkt were assessed at each

Penfluridol reduces expression of integrin α6, αV, and pFAK in a dose dependent manner

Penfluridol has been shown to reduce integrin expression at a transcriptional level in breast cancer models (10). To evaluate the role that Penfluridol plays on expression of integrin and FAK signaling in glioma models, we analyzed changes in protein levels of integrin α6, αV, and pFAK with different doses of Penfluridol treatment by western blot analysis. GBM12, GBM43, and GBM1016 were plated on a six well plate and treated with three concentrations of drug for 18 hours. Cell lysates were then collected and probed for integrin α6, αV, and pFAK. We observed that Penfluridol treatment reduced the expression of integrins α6, αV, and pFAK in a dose dependent manner in all three primary GBM models (Fig. 3a).

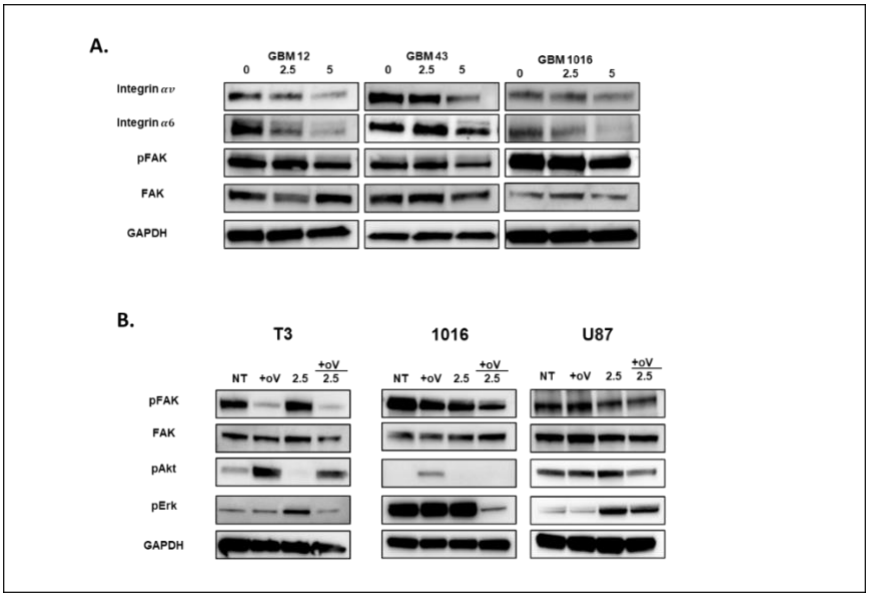

Fig.3 Effects of OV and PF on integrin expression and FAK signaling in GBM
(A) Western blot analysis in GBM cell lysates treated with PF for 18 hrs and virus +PF treatment for 24hrs (B) PF treatment alone significantly reduced integrins αv and α6 as well as FAK phosphorylation. Virus +PF treatment reduces phosphorylation of FAK as well as downstream targets such as Akt and Erk. GAPDH was used as loading control.

Combination treatment of Penfluridol and oHSV decreases activation of FAK, Akt and Erk.

FAK phosphorylation can lead to the activation of multiple downstream pathways that allow for cell proliferation. Therefore, we evaluated the effects of combining our oHSV treatment with Penfluridol. GBM1016, U87ΔEGFR, and T3 were plated on 6 well plates and treated at an MOI of 1 for one hour. Unbound virus was washed away and cells were overlaid with 2.5uM of Penfluridol. Cell lysates were collected 18 hours post infection and probed for phosphorylated FAK by western blot analysis. Decreased pFAK expression was observed in cells treated with combination of OV and Penfluridol. Additionally, we probed for phosphorylated Akt as well as phosphorylated Erk to examine the effects of the combination treatment on downstream targets in the FAK signaling pathway. We observed that the combination treatment of oHSV and Penfluridol reduced expression of both pAkt and pErk (Fig. 3b).

Penfluridol alone kills glioma cells in a dose dependent manner and when combined with oHSV has an additive killing effect.

In addition to investigating the role that Penfluridol plays in attenuating integrin and FAK signaling in glioma cells, we also examined the cytotoxicity of Penfluridol in glioma cells. U87ΔEGFR, T3, GBM28 and GBM43 were plated on 96 well plates and treated with five different concentrations of Penfluridol. Cells were treated with cell proliferation kit (Roche) reagents 48 hours post treatment, and absorbance was measured using a Synergy HT Multi-Mode Microplate reader (Biotek). Cell viability was measured as a percentage of uninfected control. We observed that Penfluridol kills cells in a dose dependent manner in all cell lines (Fig. 4a).

Additionally, we examined the cytotoxicity of Penfluridol in combination with oHSV. U87ΔEGFR, T3, GBM28 and GBM43 were plated on 96 well plates and treated with four different MOIs of oHSVQ. One hour later, cells were overlaid with three different concentrations of Penfluridol. Cells were treated with cell proliferation kit (Roche) reagents, either 24 or 48 hours post infection, and absorbance was measured using a Synergy HT Multi-Mode Microplate reader (Biotek). We observed that the individual virus and drug treatments induced cytotoxicity; however, when the two treatments were combined, we observed an additive or greater than additive killing effect in all cell lines (Fig. 4b)

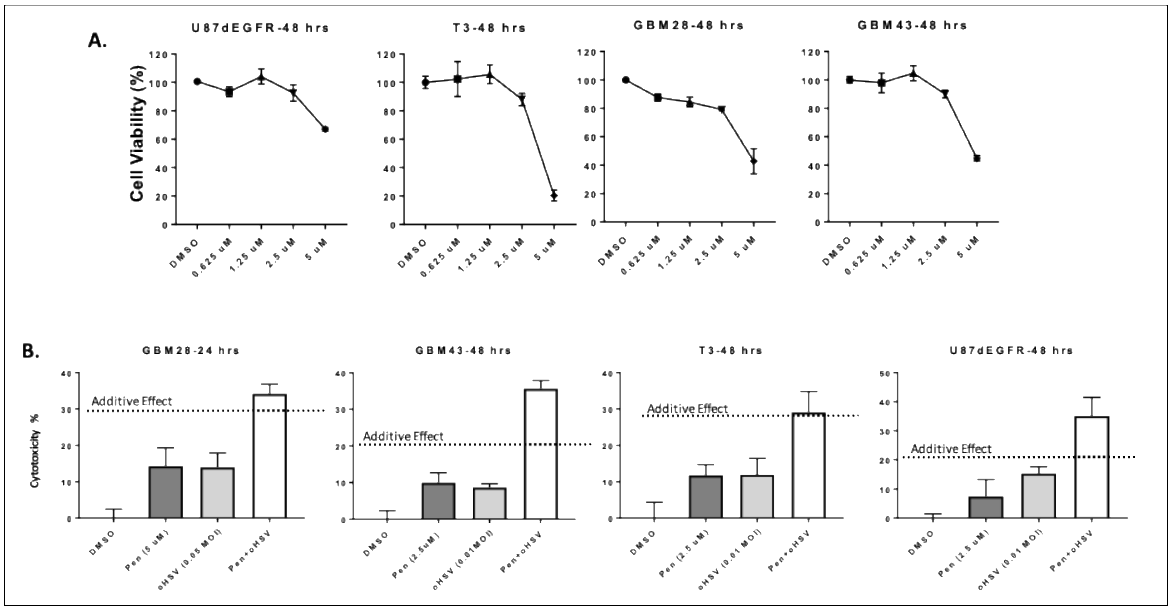

Fig.4 Effects of PF and oHSV on tumor cell killing
A) Mean % cell killing of PF alone relative to uninfected cells (B) Mean % killing of virus +PF alone relative to uninfected cells. Error bars indicate ± SD for each group. *, $P < 0.05$.

Penfluridol inhibits viral propagation at high concentrations in vitro

Since Penfluridol has an effect on integrin expression which are also important for viral entry, we examined the effect of Penfluridol on viral propagation in vitro (7). T3, GBM12, GBM28, and GBM43 cells were plated on 24 well plates and treated with an MOI of 0.5 oHSVQ. One hour later unbound virus was washed away, and cells were overlaid with five different concentrations of Penfluridol. The plate was imaged every 4 hours for 48 hours using the Cytation 5 live plate reader/imager (BioTek Instruments) to track GFP expression elicited by our GFP encoded virus. Captured images were analyzed by BioTek Gen5 version 2.06 software program, and GFP count was extracted and applied in Prism (GraphPad) to compare treatment groups. Consistent with reduced integrin expression treatment of cells with higher concentrations of PF inhibited viral spread in vitro (Fig. 5).

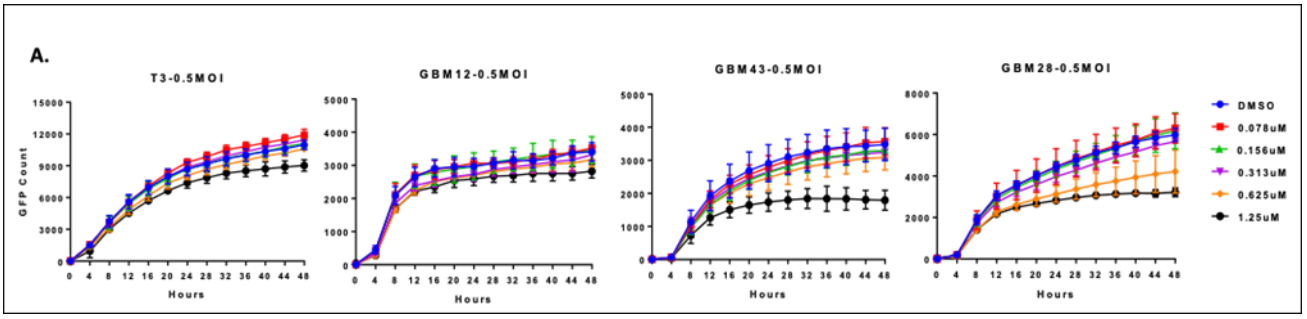

Fig.5 Effects of Penfluridol on Viral Replication *in vitro*
A) Comparison of viral kinetics/replication between oHSV and oHSV+PF. Glioma cells were infected with oHSV (MOI=0.1) and viral GFP expression was quantified for 48 hrs utilizing the Cytation 5 Live Cell Imaging.

Penfluridol does not inhibit viral replication in vivo

Based on the in vitro effects of combining Penfluridol and oHSV, we then proceeded to examine the anti-tumor effects of combining PF treatment with OV *in vivo.* Athymic nude and NSG mice were implanted with 1x10^5 GBM30 and GBM12 cells, respectively. Athymic nude mice, bearing GBM30 tumors, were treated with PBS or 1x10^5 pfu of rHSVQ1-Luc intratumorally 10 days post tumor implantation and began daily treatment of DMSO or 10mg/kg of Penfluridol the following day. NSG mice, bearing GBM12 tumors, began daily treatment of DMSO or 10mg/kg of Penfluridol 9 days post tumor implantation and were treated with PBS or 1x10^5 pfu of rHSVQ1-Luc intratumorally the following day (Fig 6a). For the three days following intracranial virus treatment, bioluminescence imaging was performed to measure viral replication via measuring virus encoded luciferase activity. Imaging data revealed that Penfluridol did not inhibit viral replication in vivo (Fig. 6b).

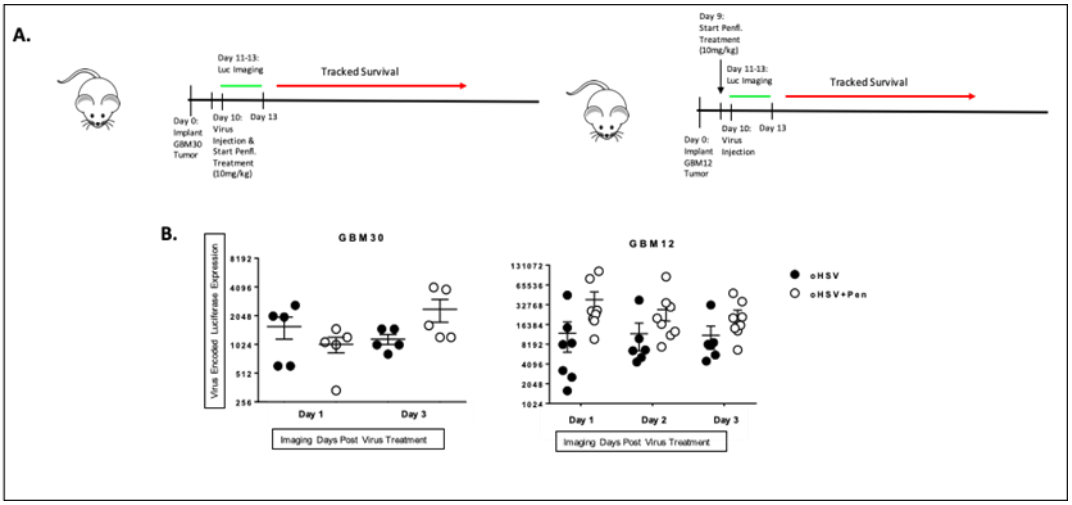

Fig.6 Effects of PF on viral replication *in vivo*
(A) Timeline of in vivo experiments (B) Mice bearing primary GBM30 and GBM12 intracranial tumors were treated with a single intratumoral injection of 2 x 10^5 oHSV-Luc. Luciferase activity was measured by IVIS imaging

Penfluridol reduces migration of macrophage and microglia cells towards infected cells

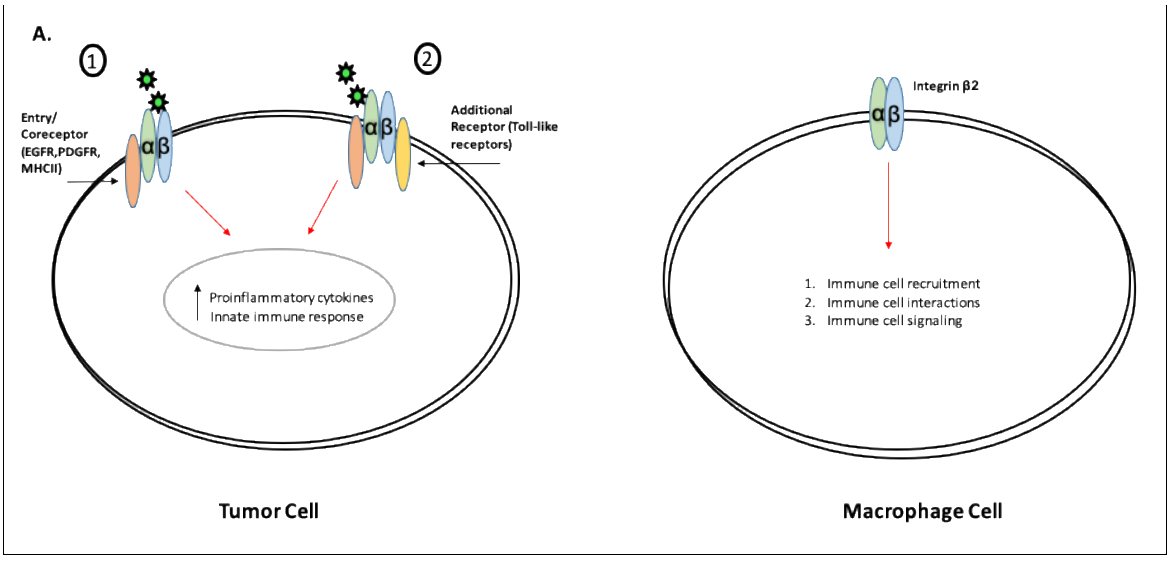

Fig.7 Graphical representation of the effects of viral entry via integrins on the tumor microenvironment.

Engagement of viral proteins with integrin receptors not only activates FAK in tumor cells, but also affects the tumor microenvironment (TME) by allowing for the transcription of pro-inflammatory cytokines and anti-viral proteins as well as activation of an innate immune

response. Additionally, in macrophages, integrin β2 is important in allowing pro-inflammatory effects to recruit and assist in immune cell migration (Fig. 7)(7, 25). Therefore, we examined the effects of Penfluridol on macrophage and microglia migration towards infected cells using a Boyden chamber assay. GBM8 cells were plated on the bottom of the Boyden chamber and treated with the indicated oncolytic viruses (black bar) at an MOI of 1 for an hour. Unbound virus was washed away and cells were then treated with or without Penfluridol. Two hours after Penfluridol treatment, serum starved murine macrophage cells (Raw 264.7) or murine microglia cells (BV2) were plated in the top chamber and allowed to migrate for 6 hours. Quantification of migrated cells were accomplished by counting the number of cells/view field that migrated to the bottom side of the transwell. Data shown are mean cells/view field ± s.d.The data showed that upon virus infection there was an increase in number of Raw 264.7 and BV2 cells which had migrated towards infected cells. However, when infected cells were treated with Penfluridol, the number of migrated macrophage and microglia cells decreased significantly (Fig. 8a).

Tumor necrosis factor (TNFα) is a proinflammatory cytokine secreted predominantly by macrophages but also other immune cells as well. TNFα binds to two TNF receptors leading to the downstream activation of both anti-apoptotic and proapoptotic pathways (11). We examined the effect of Penfluridol on TNFα secretion from macrophage and microglia cells. Raw 264.7 and BV2 cells were plated on 6 well plates and treated with Penfluridol for 24 hours. Conditioned media was collected from each triplicate well and concentrations of secreted murine TNFα was measured using the murine TNFα DuoSet ELISA kit (R&D Systems). Serial dilutions of a known concentration of purified recombinant murine TNFα were used to establish a standard curve. Quantified data showed that Penfluridol treatment reduced secretion of murine TNFα in both macrophage and microglia cells (Fig. 8b).

Combination treatment of oHSV and Penfluridol enhances survival in vivo

Based on the *in vitro* and *in vivo* data that was previously shown, we wanted to confirm whether an overall therapeutic benefit was achieved when combining Penfluridol with our OV. Thus, we tracked survival of mice bearing intracranial GBM30 or GBM12 mouse models treated with PF and/or OV. Kaplan Meier survival curves showed that the combination treatment of oHSV and Penfluridol significantly enhanced survival compared to individual treatments in both the immune deficient mouse models.

Additionally, we examined the effects of OV and PF in immune competent mouse models as well. The use of immune competent mice is beneficial as it allows us to investigate effects of the host immune system on PF and OV therapy. Both effector and suppressor immune cells can play a role in harming tumor cells or recruiting immune cells that are tumor supportive (12). In our study we are interested in the effects that OV and PF can have on both the tumor and the TME. Therefore, the use of an immune competent mouse model can help us assess these effects by examining survival and potentially immune cell populations that are present in the different treatment groups as well. We implanted 1x10^5 GL261N4 murine glioma cells into C57BL/6 mice intracranially. Mice began daily treatment of DMSO or 10mg/kg of Penfluridol 9 days post tumor implantation and were treated with PBS or 1x10^5 pfu of rHSVQ1-Luc intratumorally on day 10. Survival for mice was recorded and analyzed using a log-rank test to compare survival curves for survival data, and Cox regression model was used to evaluate the interaction between Penfluridol and oHSV on survival data. Survival in the combination treatment was enhanced even further in the immune competent mouse model compared to individual treatment groups (Fig. 9b and c).

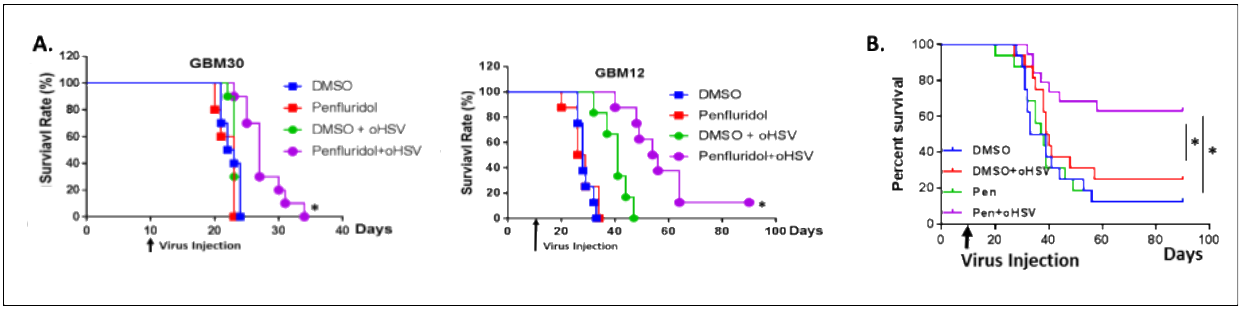

Fig.9 Effects of oHSV and PF on survival *in vivo*

(A) Kaplan- Meier survival data of GBM30-Luc and GBM12. Intracranial GBM-bearing mice were injected with oHSV by intratumoral injection. DMSO/PF (10mg/kg) were treated by daily oral gavage during study. Significant reduction in tumor growth in PF and oHSV combination-treated mice (B) Intracranial GL261-N4-bearing C57BL/6 mice were injected with oHSV by intratumoral injection and DMSO/PF (10mg/kg) were treated by daily oral gavage. Significantly enhanced mice survival rate in combination-treated mice

Discussion & Conclusion

Due to its invasive nature and volatile tumor microenvironment (TME) the need for effective therapeutics against glioblastoma (GBM) is necessary in order to improve survival in patients (2). Oncolytic viral (OV) therapy has shown promise in the lytic destruction of cancer cells, specifically in melanoma for which it is currently FDA approved as a form of therapy (4, 5). However, a concern regarding OV therapy is the risk that the virus can activate pathways that cause viral clearance and promote tumor growth. In this study, we examined the role of integrins in viral entry and how their enagagement can ultimately lead to the activation of FAK. Integrins are made up of an α and β subunit and bind extracellular matrix proteins thereby regulating cell adhesion and signaling across the cellular membrane (6). In GBM, a negative correlation has been seen between overexpression of integrins in GBM and overall survival. αVβ3 and αVβ5 integrins were first discovered to be therapeutic targets for GBM as they are not expressed on normal brain tissue but instead found solely on tumor associated endothelial cells and GBM cells. Malric *et al.*, conducted a statistical analysis based on TCGA Affymetrix data to determine the relationship between integrin expression and overall survival in GBM patients. They found that patients with primary GBM tumors that had overexpression of β1, β3, α3, α5, and αV had a decreased overall survival (6). Campadelli *et al.*, found that expression of αVβ3, αVβ6, and αVβ8, is vital in initiating virus entry (REF HERE). These three receptors bind to the gH/gL complex on the viron envelope to allow the virus to enter the cell. Additionally, αVβ6 and αVβ8 bind the gH/gL complex at a higher affinity than αVβ3. Furthermore, blocking or depletion of either β6 or β8 integrins results in the inhibition of viral entry, which emphasizes the significance of these integrins in viral entry (7). Integrin activation can ultimately lead to the activation of numerous downstream pathways that can allow for tumor growth and metastasis. One of the

major downstream targets is Focal Adhesion Kinase (FAK). Stimulation of FAK by integrins promotes what is called FAK-survival to allow evasion of cell death (13). In this paper, we examined the effects of viral infection on FAK activation at two timepoints following infection through western blotting analysis. Figure 2a shows activation of FAK 1 hour post infection as well as activation of Akt. At 8 hours post infection however, there is a decrease in FAK activation while Akt activation is sustained. This result suggests that FAK activation is temporal and that integrin-mediated viral entry can lead to activation of FAK at an early time point. Additionally, activation of Akt, a downstream target of FAK, was seen at both early and later timepoints suggesting that Akt activation may be independent of the FAK signaling pathway (13).

Since FAK activation can result in the activation of multiple downstream targets that promote cell invasion, metastasis, angiogenesis, and resistance to apoptosis (14), we investigated the use of Penfluridol (PF) , an FDA approved antipsychotic that has been shown to attenuate integrin-FAK signaling on oncolytic viral herapy (10). PF was developed in 1963 and functions as a long-acting drug by being deposited in fatty tissue after absorption in the GI tract and being slowly released over time (15). Therefore, the drug is given weekly to patients. As an antipsychotic, it functions as a dopamine antagonist by blocking the dopaminergic receptor membranes (16). The repurposing of PF as a potential cancer therapeutic is something that has previously been explored and has been shown to elicit cytotoxic effects against breast-brain metastasis and GBM, as well as downregulation of integrin $\alpha 6$ and $\beta 4$ at a transcriptional level (9, 10). From this finding, we investigated the effects that PF would have on integrin and pFAK expression in primary GBM cells. Using western blot analysis, we specifically focused on expression levels of integrin αV and $\alpha 6$ as well as activation of FAK. We saw that Penfluridol

reduces expression of both integrin αV and α6 in a dose dependent manner and also reduced activation of pFAK in a dose dependent manner. Due to the fact that virus infection can activate FAK and Akt we were interested in the effects that the combination treatment of OV and PF would have on this signaling pathway and its downstream targets. Through western blot analysis we examined the activation of FAK, Akt and Erk with OV and PF as individual treatments and as a combination treatment. In all three GBM cell lines, the combination treatment group reduced activation of FAK as well as Akt and Erk. This result illustrates the idea that PF can assist in attenuating the activation of certain signaling pathways that would normally be upregulated upon virus infection.

In addition to the therapeutic advantage that OV and PF offer as a combination treatment, we also examined the effects of both agents on tumor cell killing, virus replication and the tumor microenvironment. It has previously shown that PF has cytotoxic effects in breast-brain metastasis and GBM models (9, 10) and increases expression of cell cycle arrest and pro-apoptotic proteins (18). We assessed PF's cytotoxicity as a single agent and in combination with OV on GBM cells in our own hands by using a cell proliferation assay. Figure 4a illustrates the fact that cell killing occurs in a dose dependent manner across all cell lines that were tested *in vitro*, however this did not translate into an in vivo efficacy as a single agent. Additionally, when PF was combined with oncolytic HSV we see that both OV and PF are effective at killing cells as single agents, however when combined they display an additive, if not more than additive, killing effect. Due to the fact that PF has been shown to reduce transcriptional expression of integrins we wanted to examine the effect PF has on viral propagation. As stated earlier, integrins play a critical role in virus entry and propagation, specifically through αVβ6 and αVβ8 (7). PF has been shown to attenuate integrin α6 and β4 expression and though their targets are

not the same, we wanted to examine whether or not PF may have an effect on viral entry *in vitro*. Using the Cytation 5 live plate reader/imager (BioTek), we tracked viral propagation through GFP expression for 48 hours. The data showed that there was a reduction in viral propagation in a dose and time dependent manner in all 4 cell lines. We noticed was that viral propagation began to reduce more significantly at higher drug concentrations at 12-16 hours post infection. During the treatment, we treated the cells with virus for one hour before washing away unbound virus and overlaying with drug. Therefore, the initial virus infection could not be attenuated by PF. However, during the second infection, which would occur around 16 hours post infection, the increase in GFP was not as great in the groups treated with higher concentrations of PF. This result is consistent with the fact that PF can downregulate integrin expression and because integrins are important for viral entry; the presence of PF may play a role in decreasing viral propagation over time.

We investigated the effects of PF further by testing the drug in combination with the virus *in vivo*. Immune deficient (athymic nude and NSG) mice were implanted with GBM30 and GBM12 tumors respectively and treated with PF or virus as singular agents or treated with both agents as a combination therapy. To compare viral replication *in vivo* between virus alone and virus + PF groups, bioluminescence imaging was conducted to track viral replication for three days in both models. Contrary to what is seen *in vitro*, there is no inhibition of viral replication *in vivo* in either mouse model. The rationale behind this difference in *in vitro* and *in vivo* result could be due to the ability for PF to successfully reach the tumor at the same concentration that it was given. The blood brain barrier (BBB) plays a critical role in dictating what can and cannot reach the brain and has multiple transporters and pumps that help regulate the homeostasis of the brian. This tight regulation has been a hurdle when it comes to drug delivery to the brain (19).

ATP-binding cassette (ABC) Transporters are abundant in the BBB and assist in maintaining lipid bilayers and transporting peptides and sterols across the BBB. However, ABC transporters are of particular interest due to the fact that they have been known to play a role in the development of a multidrug resistance (MDR) phenotype (20). Overexpression of P-glycoportein, which is encoded by the MDR gene, is thought to have been an evolutionary mechanism put in place protect the brain. In fact, it was seen that knockdown of MDR1a/MDR1b in mice caused a 100 fold increase in uptake of neurotoxins (20). Although Rajan *et al* stated that they saw 72% tumor inhibition upon treatment of PF in an intracranial model and that there is BBB penetrability by PF (10), there is little evidence that illustrates how much PF actually reaches the brain. A paper published in 1979 by Grindel *et al.,* examined how PF was metabolized in rabbits, rats, dogs and humans. They examined percent dose of PF per organ, after 24 hours, by tagging the drug with a radioactive isotope and measuring radioactivity in each organ in rats. On average the percent dose of PF that reached the brain was less than 1% when given orally (5mg/kg) and through i.v (2mg/kg) (21). Another method by which we can assess how much PF has reached the brain would be to harvest the brains of PF treated mice and perform mass spectrometry analysis to determine the amount of PF present after treatment. After determining the concentration of PF that reaches the brain we can test that concentration *in vitro* to determine if it is enough to inhibit viral replication. Additionally, if PF penetration is actually limited by the BBB and its transporters, knocking down the MDR1a/MDR1b gene and tracking survival as well as comparing PF in the brain through mass spectrometry between MDR null and wild type could illustrate the limitations of drug delivery through the BBB.

We continued to investigate the role of PF by examining its effects on the tumor microenvironment (TME). Campadelli *et al.,* discuss the fact that activation of integrins upon

viral entry can ultimately lead to the activation and recruitment of immune cells and cytokines that can encourage viral clearance (7). Therefore, we examined the effects of PF on macrophage and microglial migration towards infected tumor cells. Our results showed that PF significantly reduced migration of macrophage and microglia towards infected cells and that PF treatment reduced secretion of TNFα from macrophage and microglia, implying that…. Despite these findings, it is still unclear as to whether the reduction in recruitment of macrophage and microglia is due to the attenuation of integrin and FAK signaling. To test this hypothesis, we could knock down FAK expression or block it in GBM cells and evaluate of the expression of cytokines such as TNFα and IFNγ as well as immune cell migration. If knockdown of FAK is not sufficient to suppress cytokine secretion and immune cell infiltration then it is possible that FAK is not the major pathway that regulates this response. We could also attempt to block integrin activation to see whether or not integrin activation is important in the downstream expression of proinflammatory cytokine secretion and immune cell infiltration. Additionally, the fact that PF can reduce anti-viral cytokines in the TME should increase viral replication *in vivo*. However, because it is unclear as to how much PF can successfully reach the brain, this may be the rationale behind why we did not see a significant increase in viral replication *in vivo*.

In addition to examining viral replication *in vivo* we also looked into the effects of OV and PF on overall survival *in vivo*. Three different mouse models were used in this study to track survival, athymic nude and NSG which are immune deficient and C57BL/6 which is immune competent. The results showed that the combination treatment of OV and PF significantly improved survival in both immune competent and immune deficient models compared to the individual treatments alone.. Six mice in the syngeneic immune competent model continued on to be long term surviors (survival >100 days) and were included in a rechallenge experiment.

Ultimately, there was no difference in survival between rechallenged and naïve mice and therefore we concluded that there was no formation of an immune memory response that could result in subsequent tumor rejection. However, it is possible that there is anti-tumor immunity without complete rejection of the tumor. To test this hypothesis, we can implant the same tumor model (GL261N4) into athymic nude mice and examine the therapeutic efficacy of PF in combination with OV and examine if these effects are dependent on the TME.

Since the effects of PF on the TME have not been thoroughly explored, it is important to consider the reasons why we may not have seen an anti-tumor memory response in our immune competent model to better understand the potential limitations of PF as a cancer therapeautic. Macrophages and T cells play an important role in activating an effective adaptive anti-tumor immune response (22). In cancer biology, macrophages, specifically tumor associated macrophages (TAMs), are one of the most common cells found in the TME of solid tumors and often correlate with poor prognosis in several different cancers. They are part of the innate immune response and act by blocking immune mediated clearance of tumor cells and engage directly with tumor cells to initiate pro-surival signaling pathways. Macrophages also encourage tumor progression by releasing cytokines, such as IL-6, that can activate anti-apoptotic pathways and induce angiogenesis through the production of vascular endothelial growth factor (VEGF) (23). Additionally, macrophages can differentatiate into two different phenotypes: M1 and M2. M1 macrophages respond to interferon gamma (IFNγ) and lipopolysaccharide (LPS) and are known to elicit a proinflammatory response. M2 macrophages on the other hand, respond to IL-4 or IL-13 and produce an anti-inflammatory response therefore encouraging tissue remodeling and a protumorigenic environment (24). Macrophages are known to express integrins, specifically integrin β2, which is expressed primarily on leukocytes. When interacting with their

α subunit, integrin β2 can activate three distinct functions: immune cell recruitment, immune cell interactions and immune cell signaling (25). Based on previous papers and our data, we know that PF is capable of downregulating the expression of integrins, specifically αV and α6. However, the specificity of PF in targeting integrins is unknown and therefore PF could have an effect on the expression of numerous integrins including those found on immune cells in the TME. In the case of macrophages, integrin β2 is important in allowing pro-inflammatory effects to recruit and assist in immune cell migration (25). If PF attenuates expression of integrin β2 in the immune competent mouse model this can be benficial in reducing viral clearance and therefore allowing for further viral propagation. However, PF's blockade of integrin β2 may also reduce pro-inflammatory effects and migration of immune cells thereby preventing the induction of an adaptive immune response. This second rationale may also be a contributing factor as to why we did not see a strong adaptive immune response. We can test this hypothesis by harvesting brain tissue of mice with OV, PF and OV+PF treatment and staining for specific immune cell populations using flow cytometry. Additionally, we can test the *in vitro* effects of PF on cytokine expression using an antibody array to check protein expression of numerous pro-inflammatory cytokines that recruit immune cells to the TME and also using qPCR to assess changes in gene expression of cytokine levels.

T cells are the second most common immune infiltrate in the TME and are a key player in the adaptive immune response. In the early stages of tumor development, T cells elicit an effector response to eradicate immunogenic cancer cells. Therefore a high level of effector T cell infiltration correlates with a better prognosis in numerous cancer types. These effector T cells include cytotoxic T cells, which directly destroy cells by releasing perforins and granzymes causing cell lysis, and helper T cells, which indirectly target cells by secreting cytokines to

recruit immune cells to the TME. As the tumor grows and the TME evolves, recruitment of regulatory T cells (Tregs) can occur, suppressing anti-tumor effects. Tregs are group of T cells known as suppressor cells and function by activating signaling mechansims that enhance the recruitment of immunosuppressive cytokines and molecules which ultimately leads to a decrease in activation of effector T cells (26). Similar to macrophages, T cells also express integrins which play a role in T cell signaling and differentiation. Expression of integrins on T cells can change throughout the lifecyle of the cell however, the most commonly found integrins include α4, αL, α5, α6, αV and β1, β2, β7 and β3 (27). One importance of integrins in T cells is the role they play in thymocyte differentiation. αLβ2, α4β1, α5β1 are important integrins that initiate differentiation of CD4+/CD8+ thymocytes by binding to ICAM-1, VCAM-1 and fibronectin repectively. In addition to assisting with T cell differentiation, αLβ2 on naïve T cells, can interact with endothelial cells by binding with ICAM-1 to become a "primed" form of αLβ2 ultimately causing T cell arrest and the production of matrix metalloproteinases (MMPs) (27). The interaction between αLβ2 and ICAM-1 can be beneficial for T cell migration, however, the release of MMPs into the TME can lead to tumor cell growth and metastasis. MMPs particulary play a role in the development of a pre-metstatic niche by remodeling the extracellular matrix of tumors and recruiting cells and factors that further contribute to metastatic colonization (28). As stated earlier, the specificity of PF on certain integrins is unknown, however we can test PF's effects on αLβ2 expression using western blot analysis and by examining gene expression of MMPs by doing qPCR on tumor cells cocultured with human PBMCs with and without PF treatment. If PF can reduce expression of MMPs, it is possible that PF can contribute to reducing tumor metastasis as well. On the other hand, because αLβ2 plays an important role in T cell

differentiation, the blocking of αLβ2 by PF may result in reduced T cell maturation and production.

Another aspect of this study that is important to address is the translational purpose and potential pitfalls of the use of PF as an anti-cancer treatment. As I mentioned earlier in the discussion, the repurposing of PF as an anti-cancer drug is a concept that has been explored. One thing to take into consideration when using a drug is the dosage and side effects associated with it. PF is an antipsychotic with a long half-life and, through clinical research, has a suggested dose is 40-80 mg/week to see clinical improvement (29). However, in order to exhibit anti cancer effects much higher concentrations of PF need to be used. It has been published that between 4.5-10umol/L is the IC50 for GBM(30) and in our own study we have seen that 4-5uM of PF was the IC50 dose which again are significantly higher than the doses given to patients to treat psychosis. The pitfall to using a higher concentration of PF to treat cancer patients is the neurological side effects that may be exhibited such as epilepsy, depression, dyskinesias, dystonia, and fatigue (30). When we began drug treatment with our *in vivo* models our dosing was 10mg/kg everyday until the mouse was sacrificed which is equivalent to a 50 mg dose everyday in humans. Overall, this exceeds the recommended dose even in patients with severe psychosis and can be a concern due to the neurological side effects associated with PF. Therefore, it is important that we examine the direct mechanistic effects of PF as an anti cancer drug and determine if there are ways that PF can be modified to elicit less side effects and toxicity while retaining it's capabilities as a cancer therapeutic.

As an antipsychotic PF functions by blocking dopamine receptors specifically dopamine receptor 2 (D2) (30). From here, the first question we could ask is whether or not there is relationship between dopamine receptor 2 and cancer and if so could this be the reason why PF is

effective in killing tumor cells. As far as what is known about PF as an anti cancer drug it is seen to lower transcriptional expression of integrin alpha 6, induce cell death, reduce cell proliferation and invasion and even suppress T regulatory cells. However, a direct mechanism of anti cancer activity by PF has not been proven and it's anti cancer effects were seen predominatly at concentrations that are significantly higher than those that are typically given to patients. Therefore, development of PF derivatives with lower antipsychotic effects and enhanced anti cancer effects could be highly significant. This concept is key because it is important to understand the direct mechanism of action for a drug so that it can be fine tuned to maximize therapeutic benfit while minimizing unwanted side effects. Ashraf-Uz-Zaman *et al* hypothesized that leveraging the pharmacophores activities to promote anti cancer effects and reducing the effects of PF on G protein coupled receptors (GPCRs) can make PF a safer and more effect caner therapeutic (31). Ultimately, they identified two modified compounds that exhibited less effects on the CNS and retained their anti cancer effects at a lower IC50 concentration than traditional PF. When testing these compounds *in vivo*, they treated mice with a dose of 5mg/kg for 7 days, instead of the usual 10mg/kg, and saw that one of the compounds reached the brain better than traditional PF. For the purposes of our study, we could examine whether or not these PF derivatives can elicit the same cytotoxicity *in vivo* while also enhancing viral replication instead of inhibiting it, therefore giving off a more synergistic effect. We can test this by using a cell proliferation assay and comparing killing in combination treatment groups, as well as assess viral replication with the PF derivative via our Cytation 5 machine through GFP count. Addtionally, since we hypothesize that traditional PF may have a more direct effect on the TME vs tumor cells we could also examine whether the PF derivative compound has the same effects on the TME as traditional PF and whether it improves survival *in vivo* as well.

In conclusion, our study highlights one aspect of the mechanistic effects of viral entry and infection and how this effect can be attenuated by FDA approved Penfluridol (PF). We showed that PF is sufficient at reducing integrin expression and FAK activation as well as increasing tumor cell killing when combined with oncolytic HSV. We also showed the effects of PF and OV *in vivo* by examining the combined agents' therapeautic efficacy. Overall, survival was significantly higher in the combination treated groups compared to individual treatments. Future directions for this study involve examining the effects of PF on immune cell infiltration in the TME by looking at whether or not PF attenuates integrin signaling on immune cells and to gain a better understanding as to the overall role of integrin signaling is in the TME as a tumor progresses.

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