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Propentofylline inhibits glioblastoma cell invasion and survival by targeting the TROY signaling pathway

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Abstract

Glioblastoma (GBM) is the most common primary tumor of the CNS and carries a dismal prognosis. The aggressive invasion of GBM cells into the surrounding normal brain makes complete resection impossible, significantly increases resistance to the standard therapy regimen, and virtually assures tumor recurrence. Median survival for newly diagnosed GBM is 14.6 months and declines to 8 months for patients with recurrent GBM. New therapeutic strategies that target the molecular drivers of invasion are required for improved clinical outcome. We have demonstrated that TROY (TNFRSF19), a member of the TNFR super-family, plays an important role in GBM invasion and resistance. Knockdown of TROY expression inhibits GBM cell invasion, increases sensitivity to temozolomide, and prolongs survival in an intracranial xenograft model. Propentofylline (PPF), an atypical synthetic methylxanthine compound, has been extensively studied in Phase II and Phase III clinical trials for Alzheimer's disease and vascular dementia where it has demonstrated blood-brain permeability and minimal adverse side effects. Here we showed that PPF decreased GBM cell expression of TROY, inhibited glioma cell invasion, and sensitized GBM cells to TMZ. Mechanistically, PPF decreased glioma cell invasion by modulating TROY expression and downstream signaling, including AKT, NF-κB, and Rac1 activation. Thus, PPF may provide a pharmacologic approach to targeting TROY to inhibit cell invasion and reduced therapeutic resistance in GBM.

Keywords

glioblastoma; survival; resistance; TROY; invasion

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Introduction

Glioblastoma (GBM) is a highly lethal tumor and accounts for 46.1% of all malignant primary brain and central nervous system (CNS) tumors [1]. Despite major research efforts and advances in diagnosis and treatment, the overall survival of newly diagnosed GBM patients remains very low at 14.6 months and only 5% of patients demonstrate greater than 5 year survival [1,2]. The current standard of care for treatment of GBM consists of maximal safe surgical resection, followed by 6 weeks of fractionated focal irradiation with concurrent daily temozolomide (TMZ), and then 6 cycles of adjuvant TMZ [3,4]. Unfortunately, the aggressive infiltration of GBM cells into surrounding normal brain precludes complete surgical resection. These invading glioma cells circumvent the effects of chemotherapy and focal irradiation, in part, through activation of cell survival pathways and ultimately lead to disease recurrence [5]. Therefore, a therapeutic modality that targets the invasive population of GBM cells may augment the current treatment regimen.

TROY, a member of the TNFR super-family, is a type I cell surface receptor whose expression increases with GBM tumor grade and inversely correlates with overall patient survival [6]. TROY is expressed in multiple cell lineages during embryogenesis, but its expression is restricted to the hair follicles and brain postnatally [7–9]. TROY is a susceptibility factor in nasopharyngeal carcinoma and metastatic lung cancer and its expression has been linked to the pathogenesis of multiple human malignancies [10–12]. In GBM, TROY overexpression activates Rac1 signaling in a Pyk2-dependent manner to drive migration and invasion [13]. Furthermore, increasing TROY expression in GBM cells confers resistance to TMZ and radiation via activation of the AKT and NF-κB signaling pathways[6]. Conversely, knockdown of TROY expression in GBM cells decreases cell invasion, increases chemosensitivity, and prolongs survival in GBM xenograft models [13,6].

Propentofylline (3-methyl-1-(5-oxohexyl)-7-propyl-3,7-dihydro-1*H*-purine-2,6-dione, PPF) is an atypical synthetic methylxanthine compound that has been studied extensively in preclinical models of CNS disorders and in Phase II and III clinical trials for Alzheimer's disease and vascular dementia [14–16]. Although the application of PPF in these various disorders has not yet been fully elucidated, the clinical trials revealed therapeutic efficacy, blood brain barrier permeability, and a minimal side effect profile [17]. It has been demonstrated that systemic PPF treatment decreased tumor growth in a CNS-1 rat model via modulation of microglia [18]. PPF inhibits the migration of microglia by decreasing TROY expression and suppresses the activation of downstream effector molecules, including Pyk2 and Rac1 [16].

The invasive phenotype of GBM tumors impedes effective therapy. Targeting molecular drivers of invasion would hamper the dispersion of GBM cells and enhance vulnerability to adjuvant chemo- and radiation therapy. We have previously demonstrated that decreasing TROY expression suppresses cell invasion and sensitizes GBM cells to TMZ and radiation [6]. Therefore, therapeutic targeting of TROY in combination with TMZ and/or radiation may enhance treatment efficacy and improve clinical outcome in GBM. In this study, we utilized the small molecule PPF to pharmacologically inhibit TROY in GBM cells. We

showed that PPF specifically decreases TROY protein expression in GBM cells and inhibits downstream signaling to AKT, NF-κB, and Rac1. Treatment of GBM cells with PPF did not affect cell proliferation but significantly decreased GBM cell invasion. Importantly, inhibition of TROY with PPF sensitized GBM cells to TMZ and radiation- induced apoptosis. Together, these data suggest that pharmacological inhibition of TROY with a repurposed drug, PPF, as a component of combinatorial therapy may limit invasiveness and improve clinical outcome in GBM.

Materials & Methods

Cell Culture Conditions

Human glioma cell line T98G (ATCC) was maintained in DMEM with high glucose (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) in a 37 °C, 5% CO2 atmosphere at constant humidity. The primary glioma patient derived xenograft (PDX) lines GBM10 and GBM43 were derived from patient surgical samples and maintained as flank xenografts in immunodeficient mice [19,20]. GBM10 and GBM43 flank tumor were resected, brought to single cell suspension via mechanical dissociation, and maintained in DMEM + 10% FBS for *in vitro* experiments. In the experiments with FBS stimulation, the cells were serum starved (DMEM + 0.1% Bovine Serum Albumin (BSA)) for 16 hours before stimulation with 10% FBS in DMEM.

Antibodies and Reagents

PPF, TMZ, and laminin were purchased from Sigma-Aldrich. Antibody against TROY was obtained from Abcam®. Antibodies against EGFR, TNFR1, Fn14, phospho- NF-κB, NF-κB, phospho-AKT, AKT, Cleaved PARP, α-Tubulin, and β-Actin were purchased from Cell Signaling Technology.

Western Blot Analysis

Immunoblot analysis and protein determination experiments were performed as previously described [21]. Briefly, monolayers of cells were washed in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonylfluoride and 1 mM sodium orthovanadate and then lysed in 2× SDS sample buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the BCA Assay (Pierce). Thirty micrograms of total protein was loaded per lane and separated by SDS-PAGE. After transfer, the nitrocellulose membrane (Invitrogen) was blocked with either 5% nonfat-milk or 5% BSA in TBST before addition of primary antibodies and followed with peroxidase-conjugated secondary antibody (Promega). Protein bands were detected using SuperSignal Chemiluminescent Substrate (Pierce) with a UVP BioSpectrum 500 Imaging System.

Cell Proliferation Assay

Cell proliferation was assessed by cell counts over a period of time. Briefly, 1.25 X 10^5 cells were seeded (n = 3) in 12-well plates in 1 mL of DMEM supplemented with 10% FBS and allowed to attach at 37° for 16 hrs. Subsequently, the cells were treated with media alone, 5, 50, and 500 μM PPF. After 0, 48, 96 and 144 hours of treatment, the cells were trypsinized and counted using the automated cell counter.

Cell Viability Assay

The CellTiterGlo® (Promega) assay was used to assess the cell viability after PPF treatment as previously described with minor modifications [22]. Briefly, cells were seeded at a density of 3000 cells/well (100 μL) in 96 well plates. Increasing concentrations of PPF (0.5 to 20μM) were added to the different wells (n = 8) and incubated for 72 hours at 37°C. Subsequently, 100 μL of CellTiterGlo® reagent was added to each well and luminescence was measured using Envision Reader. On all 96 well plates, wells containing vehicle only or the positive control compound MG132 (a proteasome inhibitor) were also included. Raw values were normalized on a plate-by-plate basis such that 100% cell viability was equivalent to the mean of vehicle wells and 0% cell viability was equivalent to the mean of the MG132 positive control. The normalized data was used to assess viability of glioma cells after PPF treatment.

Colony formation assay

A clonogenic assay was used to assess cell survival after radiation and TMZ treatment as described previously [23]. Briefly, 5.0×10^5 cells were seeded in 100-mm diameter culture dishes and incubated overnight at 37°C. Subsequently, cells were pre-treated with 5μM PPF for 24 hours and then either treated with 250μM TMZ for 24 hours or exposed to 2Gy radiation dose using a RS 2000 X-ray irradiator. Following combination therapy, cells were trypsinized, counted, and plated in a 6-well culture dish at densities of 100, 250, and 500 cells per well in triplicate. Cells were incubated for 12 days then fixed, stained with 0.5% crystal violet solution, and counted manually by blinded observers.

Matrigel Invasion Assay

Invasion of glioma cells treated with PPF was analyzed as previously described with minor modifications [24]. Briefly, 5.0×10^5 glioma cells were seeded in 100-mm diameter culture dishes and incubated overnight at 37°C. Subsequently, cells were serum starved for 16 hours at 37°C. Cells were then harvested, resuspended in growth factor reduced Matrigel (Becton Dickinson) (1.0 X 10^5 cells/50uL), added in triplicates to collagen-coated transwell chambers, and allowed to invade through Matrigel in presence of 10% FBS and/or 5 μM PPF. After incubation for 24 hours at 37°C, non invaded cells were scrapped off the upper side of the membrane and cells invaded to the other side of the membrane were fixed with 4% paraformaldehyde (PFA) (Affymetrix) and stained with DAPI (Invitrogen). Nuclei of invaded cells were counted in five high power fields (HPF) with a 20X objective.

Rac1 Activation

Rac1 activity assays were performed according to the manufacturer's protocol (Thermo Scientific). Briefly, 5.0×10^5 cells were seeded in 100-mm diameter culture dishes and incubated overnight at 37°C. Cells were serum starved for 16 hours at 37°C and then pre-incubated with 5 μM PPF or vehicle for 1 hour prior to 10% FBS stimulation for 2–10 min. Cell lysates were harvested and equal concentrations of protein were assessed for Rac1 activation.

Quantification of Lamellipodia Formation

Lamellipodia formation in glioma cells under PPF treatment was interrogated as described previously [25]. Briefly, glioma cells were plated onto 10-well glass slides pre-coated with 10 μg/ml laminin at the density of 3000 cells/well for 24 hours at 37°C. Subsequently, cells were serum starved for 16 hours. The cells were preincubated with 5 μM PPF or vehicle for 1 hour prior to 10% FBS stimulation for 5 min. After FBS stimulation, cells were fixed in 4% PFA, permeabilized with 0.1% Triton X-100, and incubated with Alexa Fluor® 555 Phalloidin (Invitrogen) to stain for F-actin. Slides were mounted with ProLong® reagent with DAPI and imaged using a Zeiss LSM 510 microscope. For each experimental condition, at least 12 images were randomly taken. Lamellipodia were traced using ImageJ software. For each cell, the fraction of the cell perimeter that displayed lamellipodia was calculated.

Results

PPF Suppresses TROY Expression in Glioma Cells

In recent work, Jacobs *et. al.* showed that PPF treatment decreases the expression of TROY in rat microglia *in vitro* and *in vivo* [16]. We have previously demonstrated that invading glioma cells over-express TROY and TROY expression increases TMZ and radiation resistance [13,6]. Therefore, we investigated if PPF could be utilized pharmacologically to decrease TROY expression in GBM cells. Long-term established cell line T98G was first utilized to test the efficacy of PPF. Cells were treated with increasing concentration of PPF, 1 to 20 μM, lysed, and TROY expression assessed by immunobloting. TROY expression decreased in concentration dependent manner, with significant suppression observed even at 1 μM PPF concentration (Figure 1A). We further validated these findings in patient-derived primary cell lines GBM10 and GBM43, which overexpress TROY, and showed that 5 μM PPF concentration was effective in lowering TROY expression in all glioma cells lines (Figure 1B). TROY expression also decreased in a time-dependent manner, when treated with 5 μM PPF for varying lengths of time, in both GBM10 and GBM43 cells (Figure 1C). PPF specifically decreased TROY expression in these glioma cells lines and did not affect the expression of other cell surface receptors, including the related TNFR1 or EGFR (Figure 1D). The effect on TROY expression was post-transcriptional, as PPF tretatment did not alter TROY mRNA as assessed by qPCR (data not shown). This data demonstrates that PPF decreases TROY expression in GBM cells *in vitro*.

PPF does not Affect GBM Cell Growth

Use of PPF in Phase II and III clinical trials has not been associated with any significant adverse side effects [14–16]. To determine if PPF influences glioma cell proliferation, T98G, GBM10, and GBM43 cells were treated with increasing concentrations of PPF and cell proliferation was evaluated over 144 hours. PPF did not effect the proliferation of glioma cells at doses up to 500 μM (Figure 2A). We also investigated if PPF induces glioma cell cytotoxicity using CellTiterGlo® assay with increasing concentrations of PPF for 72 hours. Treatment with PPF resulted in a negligible loss of cell viability in all three GBM cell lines (Figure 2B). These observations are consistent with our previous finding that genetically altering TROY expression does not influence GBM cell proliferation [6].

Together, these data corroborate the published reports describing the limited side effect profile of PPF and demonstrate that even at high doses, treatment with PPF does not cause toxicity in GBM cells.

PPF Sensitizes Glioma Cells to Chemo- and Radiation Therapy via Suppressing TROY-Dependent Survival Signaling

Knockdown of TROY expression with shRNA decreases TMZ resistance in GBM cells *in vitro* [6]. To corroborate these results with pharmacological inhibition of TROY expression, we tested whether the combination of PPF treatment and the current standard of care would result in enhanced therapeutic efficacy. Our results demonstrate that treatment with PPF in combination with TMZ significantly decreased the surviving fraction of T98G and GBM43 cells when compared to TMZ treatment alone (Figure 3A). Similarly, combination treatment with PPF and 2Gy radiation significantly decreased the surviving fraction of GBM cells when compared to 2Gy radiation alone (Figure 3A). To test whether the decrease in survival was due to an increase in apoptosis, we isolated lysate from T98G glioma cells after treatment with vehicle, PPF alone, TMZ alone, and PPF in combination with TMZ and immunoblotted for cleaved PARP. We found that T98G cells exposed to the combination treatment of PPF and TMZ showed an increase in cleaved PARP as compare to TMZ treatment alone (Figure 3B). Since TROY-mediated therapeutic resistance is dependent upon activation of the AKT and NF-κB signaling pathways [6], we investigated the effect of PPF on TROY survival signaling. T98G and GBM43 cells were treated with PPF, lysed, and then immunoblotted to assess the activation of AKT and NF-κB. PPF effectively decreased AKT and NF-κB phosphorylation in both cell lines (Figure 3C). These data validate that PPF inhibits TROY survival signaling pathways and augments the efficacy of TMZ in GBM.

PPF Suppresses Glioma Cell Invasion and Rac1 Activation

Our previous reports show that increased expression of TROY increased glioma cell migration *in vitro*, cell invasion *ex vivo* in an organotypic brain slice invasion assay, and stimulated the migration of normal astrocytes *in situ* [13,6]. Therefore, we next examined whether PPF inhibited TROY dependent glioma cell invasion. T98G, GBM10, and GBM43 glioma cells were treated with PPF, and using a Matrigel invasion assay, we demonstrated that treatment with 5 μM PPF significantly inhibited glioma cell invasion *in vitro* (Figure 4A). TROY-mediated invasion is induced, in part, through activation of Rac1, and our results showed that PPF inhibited Rac1 activation in T98G cells (Figure 4B) [13].

Rac1 plays a key role in the cytoskeletal rearrangements, including lamellipodia formation, which is associated with invasion [26,27]. Likewise, we investigated an essential step in cell motility, lamellipodia formation and membrane ruffling. We evaluated lamellipodia formation after PPF treatment in T98G, GBM10, and GBM43, cells, and showed that PPF significantly decreased lamellipodia formation and membrane ruffling (Figure 5). These data are consistent with our previous data that shRNA depletion of TROY expression decreases GBM invasion and establish that pharmacologically decreasing TROY expression inhibits the invasive potential of GBM cells [6].

Discussion

The ability of GBM cells to invade the dense parenchyma of normal brain tissue is a key factor in the evasion to local therapies, including surgical resection and chemotherapy. Therefore, the identification of a small molecule that limits dispersion of GBM cells would enhance surgical removal of the bulk tumor, limit adverse effects on brain function, and decrease tumor recurrence. Moreover, the invasion process upregulates cell survival pathways and increases the resistance of invasive cells to therapeutic agents. Previously, our lab demonstrated that TROY is overexpressed in high-grade glial tumors when compared to non-neoplastic brain, negatively correlates with overall patient survival, and stimulates invasion through Pyk2-Rac1 signaling [13,6]. Collectively, these data suggest that TROY expression and signaling is a potential target to limit GBM invasion and enhance therapeutic sensitivity. To date, no compounds have been developed for clinical use that target invasion in GBM.

In the recent literature, there has been an emergence of interest in the repurposing of FDA approved drugs for the treatment of GBM and other cancers[28,29]. Propentofylline has been shown to decrease TROY expression and migration in rat microglia [16] and has been used in phase II and III clinical trials for vascular dementia and Alzheimer's disease [30,14,15]. In the current study, we evaluated the effect of PPF on TROY dependent glioma cell invasion and survival. We demonstrated that PPF decreased TROY expression in GBM cells and suppressed its invasion *in vitro* without any significant direct toxicity to the glioma cells. Moreover, PPF inhibited TROY signaling pathways, including Rac1 activation. Rac1 is a known mediator of migration and invasion and siRNA knockdown of Rac1 decreases TROY mediated glioma cell invasion [26,27,13]. The current data indicate that PPF efficiently attenuated TROY protein expression and the associated invasive GBM cell phenotype. Mechanistically, it is unknown how PPF suppresses TROY protein expression, however the mechanism of suppression is currently under investigation.

Activation of the invasive phenotype in glioma cells mitigates apoptosis induced by cytotoxic-agents. Invasive cells strongly activate survival pathways that render these cells resistant to apoptosis [31]. Therefore, targeting invasive pathways may have a dual beneficial effect by limiting dispersion and enhancing sensitivity to chemotherapy [32]. Increased TROY expression in GBM enhances resistance to both radiation and TMZ via activation of the AKT and NF-κB signaling pathways [13,6]. Our data show that PPF treatment decreased TROY expression and concomitantly decreased the phosphorylation of AKT and NF-κB, demonstrating that PPF inhibits TROY survival signaling. Moreover, the combination of PPF and TMZ or radiation resulted in a significant decrease in survival compared to single-agent therapy alone, suggesting synergism between pharmacological inhibition of TROY and treatment with cytotoxic agents. TROY has also been implicated in the pathogenesis of other tumors, which suggests inhibition of TROY may be a therapy in other malignancies [12,11]. Together, these results suggest that PPF may be a potential adjuvant medication that will increase tumor vulnerability and garner improved therapeutic response in patients.

One of the major challenges in the translation of newly developed targeted therapies for the treatment of GBM is the limited drug permeability through the blood brain barrier (BBB) [33]. However, PPF is capable of penetrating the BBB, has been already FDA approved, shows very low toxicity profiles in patients, and displays efficacy in suppressing TROY protein expression in glioma cells *in vivo* [15,14]. Therefore, PPF could be easily translated to the clinic as an adjuvant therapy in combination with standard of care for treatment of GBM patients.

In summary, we demonstrated that targeting TROY expression and signaling with PPF is an encouraging strategy for increased treatment efficacy in GBM. Inhibition of TROY expression limited the invasion of GBM cells and decreased resistance to TMZ and radiation. Therefore, utilizing PPF, a small molecule inhibitor of TROY, in conjunction with TMZ and radiation warrants testing in murine xenograft models to determine its potential as a therapeutic regimen for GBM patients. These studies are currently under investigation.

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Abbreviations

GBM	glioblastoma
PPF	Propentofylline
TMZ	temozolomide

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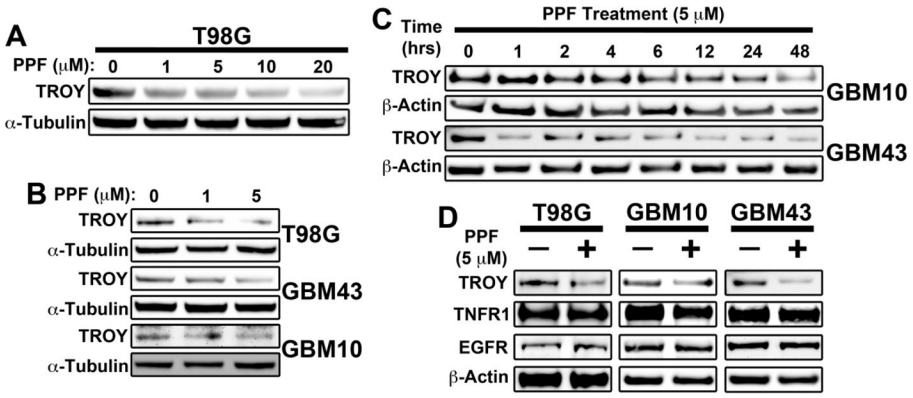

Figure 1.

PPF treatment decreases TROY expression in GBM cells. (A) Western blot analysis of TROY expression in T98G cells. Cells were treated with PPF at indicated concentrations for 6 hrs, lysed, and cell lysates immunoblotted with anti-TROY antibody. Immunoblotting of α–Tubulin in cell lysates is included as a loading control. (B) Western blot analysis of TROY expression in T98G, GBM43, and GBM10 cells. Cells were treated with PPF at indicated concentrations for 24 hrs, lysed, and cell lysates immunoblotted with anti-TROY and α–Tubulin antibodies. (C) Western Blot analysis of TROY expression in GBM10 and GBM43 primary glioma cells. Cells were treated with 5 μM PPF, lysed at the indicated time-points, and the lysates immunoblotted with an anti-TROY antibody. Immunoblotting of β-Actin in cell lysates is included as a loading control. (D) Western Blot analysis of T98G, GBM10 and GBM43 glioma cells treated with 5 μM PPF for 24 hours. Lysates were immunoblotted with anti-TROY, anti-TNFR1, anti-EGFR, and β-Actin antibodies.

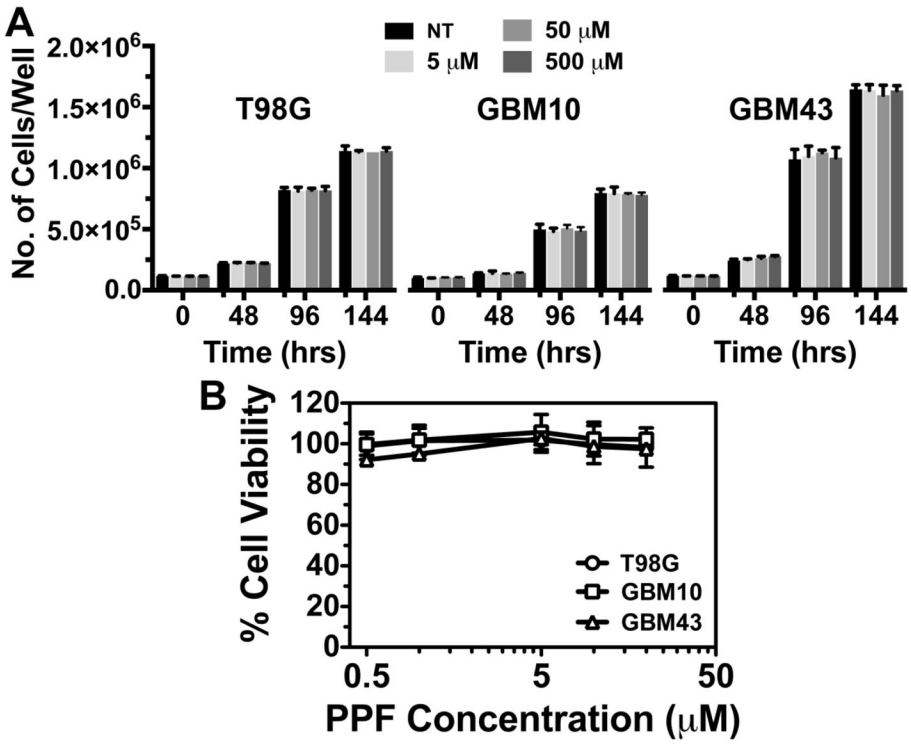

Figure 2.

PPF does not affect the proliferation in GBM cells. (A) T98G, GBM43, and GBM10 cells were incubated with increasing concentrations of PPF (0, 5, 50, and 500 μM). After 0, 48, 96 and 144 hours of treatment, cell were trypsinized and counted using an automated cell counter. (B) GBM43, GBM10, and T98G glioma cells were treated with increasing doses of PPF (0.5, 1, 5, 10, and 20 μM) in triplicate. The Cell Titer Glo (Promega) reagent was used to measure survival. Raw values were normalized on a plate-by-plate basis such that 100% cell viability was equivalent to the mean of vehicle wells and 0% cell viability was equivalent to the mean of the MG132 positive control. The normalized data was used to assess viability of glioma cells after PPF treatment.

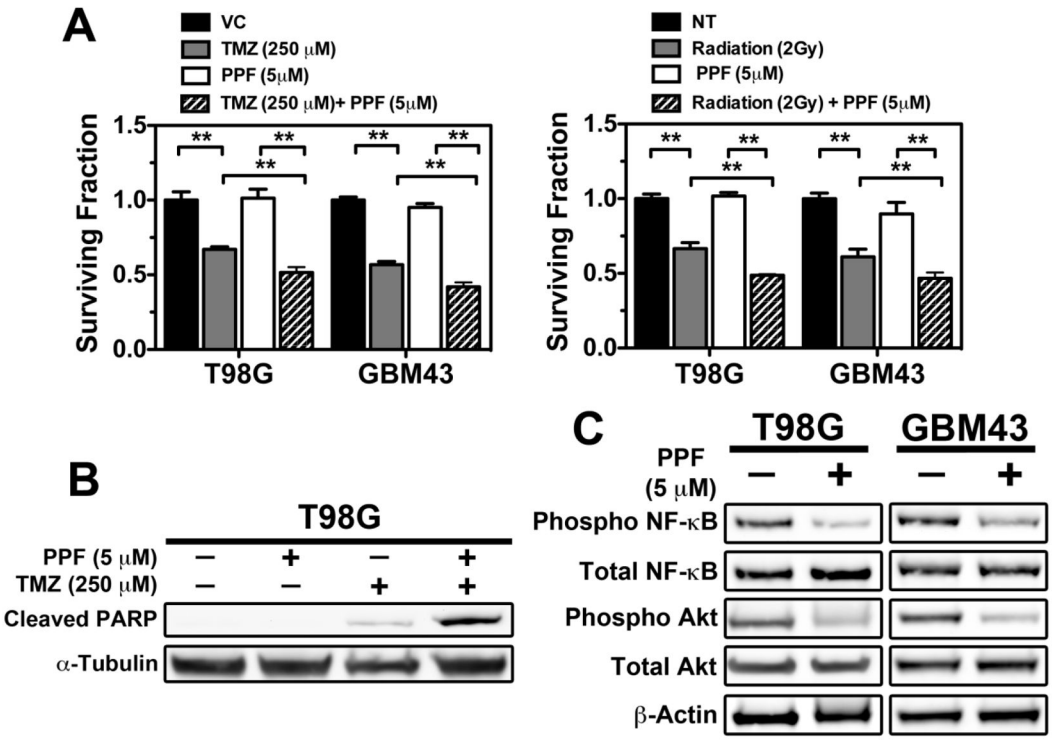

Figure 3.

PPF sensitizes GBM cells to TMZ and radiotherapy (A) A clonogenic assay was used to assess T98G and GBM43 cells survival after TMZ and radiation treatment. Cells were pre-treated with 5μM PPF for 24 hours, and then either treated with 250 μM TMZ for 24 hours or exposed to 2 Gy radiation. Graph depicts the surviving fraction in the treated cells compared to vehicle (VC) treated or non-treated (NT) cells, **p<.01. (B) T98G glioma cells were treated with vehicle, PPF (5 μM), TMZ (250 μM), and PPF in combination with TMZ. TMZ-induced apoptosis was assayed by immunoblot analysis of cell lysates with an antibody to cleaved PARP. Immunoblotting for α-Tubulin was included as a loading control. (C) T98G, GBM10, and GBM43 cells were treated with PPF (5 μM), lysed, and then immunoblotted to assess the activation of AKT and NF-κB. Immunoblotting for β-Actin is included as a loading control.

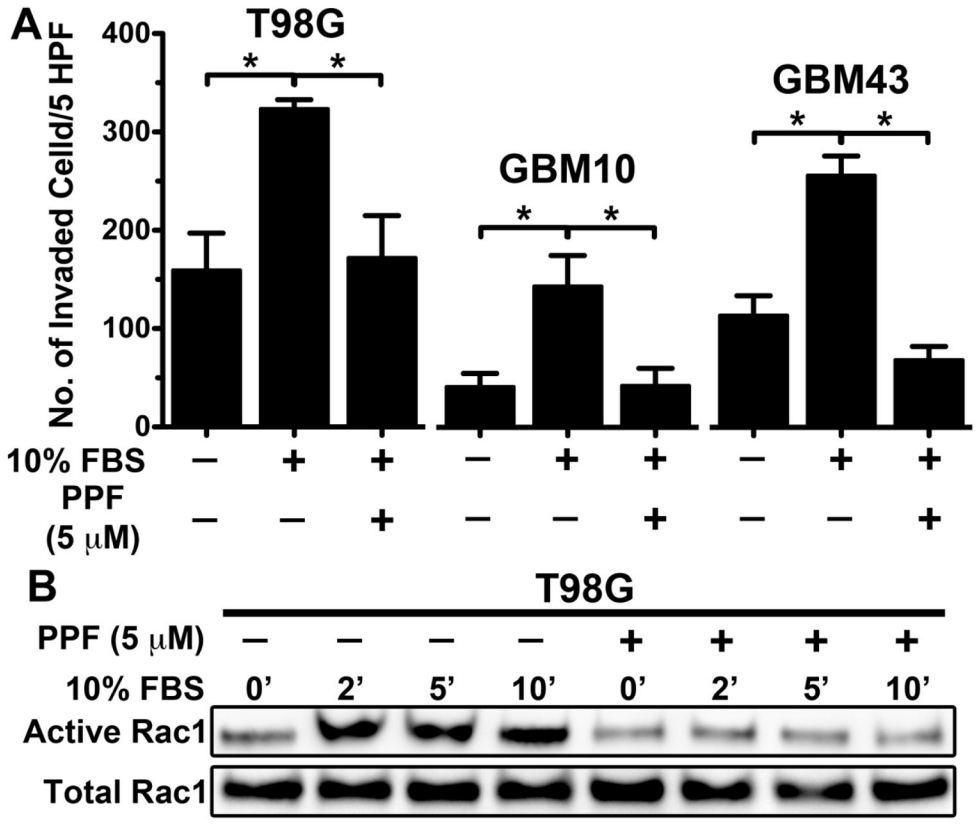

Figure 4.

PPF suppresses GBM cell invasion and Rac1 activation. (A) T98G, GBM10, and GBM43 glioma cells were treated with 5 μM PPF and invasion was assayed over 24 hours utilizing a Matrigel invasion assay, *p<.05. (B) T98G glioma cells were serum starved, pre-incubated with 5 μM PPF or vehicle for 1 hour, and then stimulated with 10% FBS for 2–10 mins. Cell lysates were harvested and equal concentrations of protein were assessed for Rac1 activation.

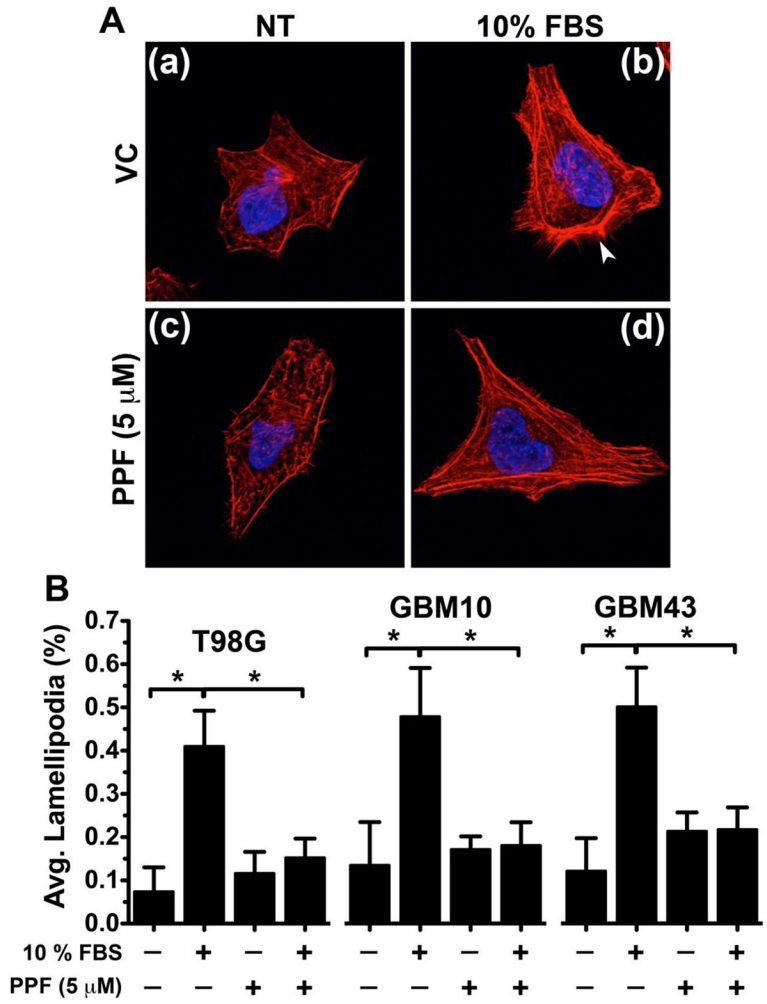

Figure 5.

PPF suppresses GBM cell membrane ruffling. (A) GBM43 cells were preincubated with 5 μM PPF or vehicle for 1 hour prior to 10% FBS stimulation for 5 min. After FBS stimulation, cells were fixed, permeabilized, and stained for F-actin. For each experimental condition, at least 12 images were taken randomly. Arrowhead indicates membrane ruffles. (B) Graph depicts the average lamellipodia in T98G, GBM10, and GBM43 cells in the presence or absence of 10% FBS with or without 5 μM PPF as indicated. Lamellipodia were traced using Image J software. For each cell, the fraction of the cell perimeter that displayed lamellipodia was calculated, *p<.05.