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Neil R. Patel Date

Antitumor Effects of Progesterone Alone and in Combination with Temozolomide Against Neurogenic Tumors

By

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Antitumor Effects of Progesterone Alone and in Combination with Temozolomide Against Neurogenic Tumors

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Advisor: Dr. Fahim Atif, PhD

An abstract of

A thesis to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Bachelor of Science/Master of Science Biology

2014

Abstract

Antitumor Effects of Progesterone Alone and in Combination with Temozolomide Against Neurogenic Tumors

By Neil Rajendra Patel

We investigated the antitumorgenic effects of the hormone progesterone (P4) against neurogenic human tumor cells *in vitro*. First, natural P4 and medroxyprogesterone acetate (MPA), a synthetic progestin, were tested for their cytotoxic effects in neuroblastoma (SK-N-AS) cells. Cell death was measured and we observed that P4 was unable to elicit significant cell death during a 72-hour single exposure. However, repeated 3- and 6-day exposures of P4 at high concentrations (20, 40, 80 μM) significantly ($P<0.05$) reduced the viability of SK-N-AS cells. Interestingly, MPA was unable to greatly reduce cell viability regardless of exposure duration. We then investigated the antitumor effects of P4 alone and in combination with Temozolomide (TMZ), a chemotherapeutic agent used to treat glioblastoma multiforme (GBM), the most aggressive of neurogenic cancers. We exposed GBM cells (U87MG) to P4 and/or TMZ either as a single exposure for 72 hours or repeated exposures for 3 and 6 days. Again, single exposures to either drug were unable to induce cell death. At high concentrations (20, 40, 80 μM) with repeated exposure, P4 significantly ($P<0.05$) reduced the viability of U87MG cells. Repeated TMZ exposures also demonstrated some significant reduction in U87MG cell viability at high concentrations (25, 50, 75, 100, 200 μM). Next, we combined TMZ with P4 to examine whether P4 would enhance the cytotoxic effects of TMZ against U87MG cells following repeated exposures. We used a concurrent drug exposure strategy for 3 and 6 days and observed that P4 enhanced the cytotoxic effects of TMZ in combination as compared to TMZ alone but the combination was still less effective than P4 alone. These findings suggest that P4 alone is a potent anti-tumor agent at high concentrations and enhances the cytotoxic efficacy of TMZ in combination in U87MG cells. Furthermore, a combination of the two drugs was also most effective in reducing GBM cell migration. Finally, we examined the modulatory effect of P4 and TMZ alone or in combination on the EGFR/PI3K/Akt/mTOR signaling pathway. Western blot data suggest that P4 alone or in combination suppresses this signaling in U87MG cells and thereby suppresses cell proliferation as evidenced by reduced expression of proliferative cell nuclear antigen (PCNA).

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My entire educational career would not have been possible without the unwavering support from my mother, Jayshri Patel, my father, Rajendra Patel, and my brother, Sunny Patel. You are the reasons I have the opportunity to challenge myself.

And lastly, to all my friends who encouraged me to pursue my passions, especially April Yang: Thank You.

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INTRODUCTION

A growing and simultaneously aging world population is contributing to an expansion in the global burden of cancer. A study by the World Health Organization (WHO) found cancer to be the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. Projected global deaths due to cancer far surpass other non-communicable diseases [2].

Across the age spectrum, deaths due to brain and nervous system cancers increased 47.8% between 1990 and 2010 [3]. Neuroblastoma, an embryonal tumor of the autonomic nervous system, is the most commonly diagnosed cancer in the first year of a child's life [4]. Though advances in therapy have produced a 74% survival rate, those with a more high-risk form of the disease have shown modest improvement in spite of an escalation of treatment intensity [5].

Another, far more malignant, neurogenic neoplasm is glioblastoma multiforme (GBM), a WHO grade IV glioma, the most potent grade. No safe and effective treatment exists for this complex and clinically challenging condition. Despite the utilization of cutting edge preoperative and intraoperative neuroimaging during surgical resection, in addition to advances in radiotherapy with concomitant chemotherapy and adjuvant chemotherapy, the median survival for GBM patients is still just 14 months [6]. Two agents are currently used as the standard-of-care treatments for GBM, Gildel, dime-sized carmustin-containing wafers administered to the resection cavity at the time of surgery [6], and

Temozolomide (TMZ), an orally administered alkylating agent. The two drugs separately increase survival by 2 months, but their application is accompanied with severe side effects [6]. Even with the most rigorous treatment regimens, GBM tumors have a high rate of recurrence in virtually every patient, leading to the final mortality rate of close to 100% [7].

Current treatments have several limitations. Surgical intervention is an essential initial step in treatment; it is often used to provide tissue specimens for histological confirmation and ultimately for gross-total resection. Accessibility for surgery is greatly hindered due to tumor location. In addition, GBM's aggressive invasive properties allow for disruption and infiltration of physical barriers such as basement membranes and cell junctions [8]. The penetration into adjacent brain parenchyma makes complete surgical removal almost impossible.

Chemotherapy is another area for improvement. The blood brain barrier's (BBB) natural protection against therapeutic agents poses an added immediate challenge of accessibility. Further, more aggressive treatments adopted to overcome GBM's insensitivity have resulted in severe side effects and complications. For example, in a randomized study exploring the efficacy of Gildel implantation during repeated resection, Brem, *et al.* reported the following complications: serious intracranial infections (2.2%), postoperative seizures, and edema that required steroid medications [9].

The final component of treatment is radiotherapy, and although advances have been made in this noninvasive procedure, various issues remain to be solved. Stereotactic radiosurgery is a method of localized irradiation often used

on small recurrent GBM lesions. Various risks are associated with the method including hydrocephalus, radiation-induced necrosis, edema, and mass effect [10]. These side effects coupled with the limitations of radiation dose due to cranial location of the tumor make complete irradiation especially difficult.

The current treatment hurdles elucidate why GBM's recurrence is virtually inevitable. As tumor progression takes place, the residual tumors tend to behave even more aggressively, causing peri-tumoral brain edema, a major factor for patients that is negatively correlated with survival [11].

Although today's treatment regimens allow patients to live out extra months of their lives, they often come at a great price. In a study of 306 newly diagnosed GBM patients, McGrit *et al.* found 15 patients (5%) who developed a new language deficit and 19 patients (6%) who developed a new motor deficit following surgery [12]. These surgically acquired neurologic deficits, coupled with side effects from chemo- and radiotherapy, clearly have an impact on a patient's quality of life.

Thus, there is an urgent need for further agents that can combat these neurogenic cancers. The optimal therapeutic would be one that: 1) permeates the BBB, 2) selectively kills tumor cells, thereby sparing healthy tissue, 3) targets multiple mechanisms of tumor progression, and 4) decreases edema and mass effect. A synergistic combination of drugs may allow cancer cell death to be maintained or enhanced while using lower doses of harmful chemotherapeutics. The reduction of chemotherapy could decrease the harmful side effects of cancer treatment.

One such agent may be the natural and pleiotropic hormone progesterone (P4). P4 is an important gestational hormone that protects the fetus from immune inflammatory attack during gestation. The outer ring of placenta cells is extremely aggressive, behaving much like tumor cells as they invade the uterine wall and tap into the mother's blood vessels. P4 plays a pivotal role along with other cell cycle machinery to counter abnormal growth during mitosis and meiosis [13]. It is important to note the distinction between natural P4 and its synthetic progestin counterparts. Though both may be classified as progestogens, their metabolic differences have been documented to cause varying effects in a biological system [14].

P4's ability to pass quickly through the BBB is central to its therapeutic potential. Years of preclinical research in our laboratory have focused on investigating P4 in the treatment of traumatic brain injury (TBI) and stroke. Stein *et al.* found that post-trauma treatment with P4 rapidly passes the BBB and decreases the cerebral edema that often accompanies TBI [15]. Furthermore, P4 conveyed evidence of efficacy and safety through two independent Phase II trials [16,17] with no serious adverse events due to treatment. Two independent Phase III multicenter clinical trials for P4 treatment of TBI are nearing completion.

Oncology is another area where researchers have explored the therapeutic effects of P4. The hormone has proven its anti-proliferative and apoptotic effects on breast, endometrial, and ovarian cancers *in vitro* and *in vivo* [18,19,20]. In support of the hormone's pleiotropic qualities, a recent microarray study reported that endometrial tumor cells exposed to high-dose P4 lead to

changes in the expression of 247 genes, including many involved in the control of the cell cycle, proliferation, and differentiation [21]. Atif *et al.* reported that high doses of P4 were capable of significantly impeding neuroblastoma tumor growth *in vitro* without inducing any cell death in healthy primary cortical neurons or human fibroblasts [22]. The same study attained a significant reduction in tumor size (~50%) with a more biologically sensitive *in vivo* model.

In the present study, we investigated the anti-tumor effects of P4 against the growth of human neuroblastoma (SK-N-AS) and GBM (U87MG) cell lines *in vitro*. We addressed the following questions: (1) Does P4 reduce tumor cell viability in U87MG and SK-N-AS cells? (2) Do natural and synthetic P4 have the same anti-neoplastic properties? (3) What is the most effective dose of P4? (4) Does TMZ reduce GBM cell viability *in vitro*? (5) Is GBM cell viability further reduced when P4 is combined with TMZ? (6) Is GBM cell migration altered with P4 and/or TMZ treatment? (7) What are possible mechanisms of action?

Cell death was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A scratch test was performed to observe changes in GBM migration following drug exposure. In order to elucidate potential mechanisms of action, we chose to study the effect of P4 and TMZ alone or in combination on the EGFR/PI3K/Akt/mTOR signaling pathway, which enhances tumor cell proliferation and is highly active in GBM. A Western blot was also performed to record the expression of a cell cycle and cell proliferation marker, proliferative cell nuclear antigen (PCNA). Together, altered expression of

these proteins constitutes one dimension in which our proposed treatment methods may be able to influence GBM progression.

METHODS

Cell culture

Human neuroblastoma (SK-N-AS) and glioblastoma (U87MG) cell lines were purchased from ATCC (Manassas, VA). As directed by the manufacturer, cells were cultured for experimentation in multiwall plates maintained at 37ºC under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% Fetal Bovine Serum (FBS) and antibiotics. Cells experimented on were kept below 25 passages and split every 5-6 days upon sub-confluence.

Experimental design for *in vitro* cell death studies

Tumor cells (SK-N-AS, U87MG) were seeded (0.5 x 10^5 cells/well) in a 24-well plate, allowed to grow till sub-confluent, and kept under starvation overnight prior to experimentation in incomplete (serum free) DMEM medium. P4 (P3972; Sigma, St. Louis, MO), MPA (M1629; Sigma), and TMZ (34219; Sigma) stocks were prepared in dimethylsulfoxide (DMSO) and further diluted in incomplete culture medium. DMSO final concentration was kept <5 µL/ mL. Cells were exposed to different concentrations of P4 (0.1 [P0.1], 1 [P1], 5 [P5], 10 [P10], 20 [P20], 40 [P40] and 80 [P80] µmol/L), MPA (0.1, 1, 5, 10, 20, 40 and 80 µmol/L), or TMZ (5 [T5], 10 [T10], 25 [T25], 50 [T50], 75 [T75], and 100 [T100] µmol/L) for either single or multiple exposures. For single exposures, each drug was added once at the start of the trial and cell viability was measured after 72 hours. Culture medium was replaced everyday in the multiple exposure model with the

respective drug (P4, MPA, or TMZ) added daily for 3 and 6 days. Cell viability was calculated on days 4 and 7 respectively.

GBM cell viability with combinatorial drug treatment

GBM cells were seeded (0.5 x 10^5 cells/well) in a 24-well plate, allowed to grow till sub-confluent, and kept under starvation overnight prior to experimentation in incomplete DMEM medium. Cells were exposed to P4 and TMZ concurrently. For concurrent exposures, P4 and TMZ (P5, P80, T100, P5+T100, P80+T100) were added to GBM cells daily upon incomplete medium replacement. This treatment schedule was repeated for 3 and 6 days with cell viability assessed on day 4 and day 7, respectively.

MTT assay

Neuroblastoma and GBM cell viability was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In the reduction assay, 15 µL of MTT solution (5 mg/mL phosphate- buffered saline [PBS]) was added to each well and incubated at 37°C for 4 hours. The blue formazan crystals precipitate as a result of mitochondrial activity in viable cells. The crystals are unable to escape healthy living cells due to cell membrane impermeability. The crystals were then solubilized with 0.5-1.0 ml of DMSO at which point the intensity of the blue coloring of the formazan solution is proportional to the number of surviving cells. Absorbance was recorded at 570 nm with a microplate reader.

PI staining

Propidium Iodide (PI) staining of neuroblastoma cell cultures was performed as described previously [23]. The intercalating agent binds to DNA, after which its fluorescent properties are enhanced between 20 and 30 fold [24]. Membrane impermeability obstructs the PI stain from entering viable cells; instead the PI enters only dead or dying cells that have a compromised cell membrane. Following repeated P4 exposure for 6 days, the stain was executed as a qualitative measure of cell death, cell membrane damage, and changes in nuclear morphology. Cells were incubated for 1 min with 0.02 mg/mL PI (stock solution 1 mg/mL, 1:50) in medium with gentle shaking and rinsed once with PBS. Conditioned medium was reapplied and phase contrast and fluorescent pictures were taken immediately with an inverse fluorescence microscope attached to a digital camera.

Cell Migration

GBM cells were seeded into in a 12-well plate (0.7×10^5 cells/well), allowed to grow till sub-confluent, and kept under starvation overnight prior to first exposure in incomplete DMEM. After dosing cells with P4 and/or TMZ, a scratch/wound was formed with a 200-µl pipette tip and the cells were kept in an incubator for the next 24 hours. Photographs were taken at 0 and 24 hours post-wound formation.

Western blot analysis

Western blot was performed as described by Atif *et al.* [22]. GBM cells were seeded ($5.0x10^5$) into petri dishes and allowed to grow in complete medium until confluent. Tumor cells were starved in incomplete medium overnight, then exposed to P4 and/or TMZ (P5, P80, T100, P5+T100, P80+T100) either as a single or a multiple exposure. Cells were exposed to the drugs for 24 hours during single exposure experiments. For multiple exposures, cells were treated for 3 days with medium and drugs added daily. In both instances, following treatment, cells were scraped from the petri dishes and protein was extracted using RIPA extraction buffer kit (Santa Cruz Biotechnology, Santa Cruz, CA**)** with protease inhibitors. Protein concentration in each sample was determined utilizing a bicinchoninic acid (BCA) microplate protein assay (23225; Pierce, Rockford, IL). Protein samples (50 µg) were separated under reducing and denaturing conditions using 4-20% acrylamide Criterion gel (BioRad, Hercules, CA) at 200V for 1 hour then transferred to a polyvinylidene difluoride (PVDF) membrane at 100V for 35 min. Non-specific binding sites on the membrane were blocked with 5% non-fat dry milk in PBS-T (PBS containing 0.05% Tween-20). The membrane was then probed with the following primary antibodies overnight at 4°C: Akt (#9272), Phospho-AKT (#9271S), purchased form Cell Signaling Technology (Danvers, MA); PCNA (#SC-56), epidermal growth factor receptor (EGFR; #SC-03), purchased from Santa Cruz Biotechnology; mTOR (#32028) from Abcam Inc. (Cambridge, MA); and β-Actin (AC74) from Sigma. Then, membranes were incubated in their respective horseradish peroxidase–

conjugated secondary antibodies. Blots were developed after adding a chemiluminescent substrate (Pierce) for 5 minutes. Chemiluminescent bands were identified on a Kodak autoradiography film in a darkroom followed by density measurements using NIH ImageJ software.

Statistical Analysis

The statistical analyses of data were performed with one-way analysis of variance (ANOVA) followed by an LSD test to compare several treatment groups to a single control group. The significance of results was set at $P < 0.05$.

RESULTS

[A]. Effects of P4 and MPA Treatment on Neuroblastoma Cell Viability

Single Exposure to P4 in SK-N-AS cells

A single 48-h exposure to P4 at a range of concentrations (0.1, 1, 5, 10, 20, 40 and 80 µmol/L) produced a U-shaped response curve for cell viability in SK-N-AS cells. There was significant ($P < 0.05$) loss at 5, 10 and 20 µmol/L, but other concentrations did not induce cell death (Figure 1).

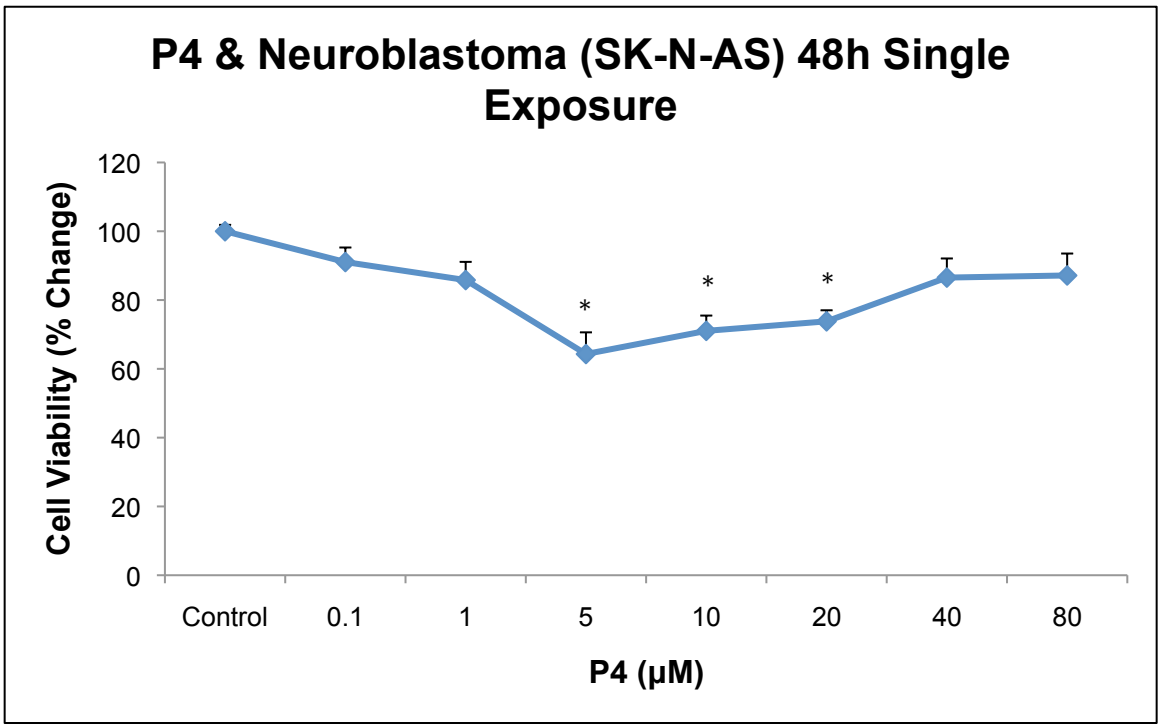

Figure 1. Short exposure effects of P4 against neuroblastoma. Cells were exposed to different concentrations of P4 applied once for 48h. The stock solution of P4 was prepared in DMSO (final concentration <5 µL/mL medium), then further diluted in culture medium. Cell death was measured by MTT assay. Data are expressed as means ± SE (n=9) from three independent experiments. Significant difference: *P<0.05 compared with vehicle group. N= 12.

Repeated P4 exposure in SK-N-AS cells

SK-N-AS cells were exposed to varying concentrations of P4 for 3 and 6 days of treatment (Figure 2). Maximum cell death was observed at the highest concentration tested (80 µmol/L). It is important to emphasize that in an identically designed study by Atif *et. al.,* repeated exposures of P4 for 3 and 6 days did not produce any death in either primary cortical neurons or HFF-1 cells; instead P4 showed significant proliferative effects in HFF-1 fibroblasts at 5, 10 and 20 µmol/L concentrations over the 6 days of exposure [22].

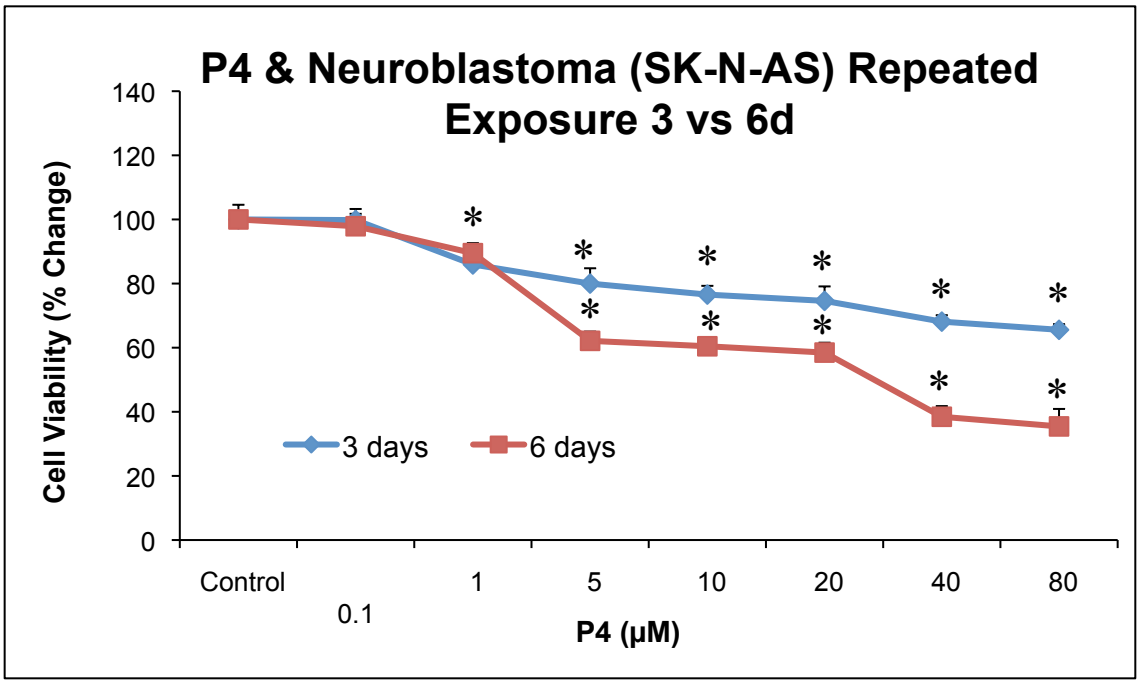

Figure 2. Inhibitory effects of P4 against neuroblastoma. Repeated 3 and 6 day P4 exposures of varying doses are compared on the basis of cell death measured by MTT assay. Culture medium containing P4 was replaced daily. Data are expressed as means ± SE (n=12) from three independent experiments. Significant difference: **P*<0.05 compared with vehicle group.

PI staining of SK-N-AS cells

Cellular uptake of PI stain causes red fluorescence and is an indication of reduced cell viability. In Figure 3, the strength of red fluorescence qualitatively amplifies as the P4 dose is increased. The data are consistent with cell viability studies quantified using an MTT assay in this work.

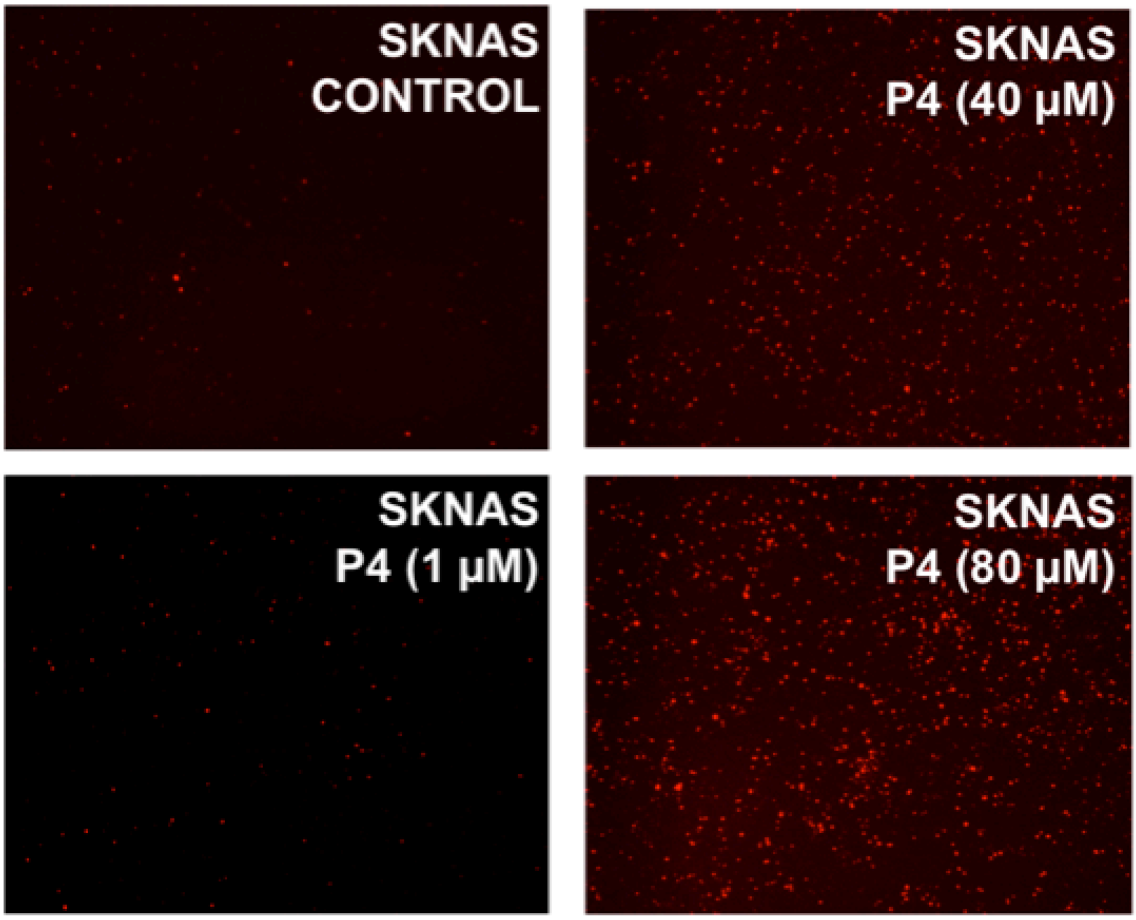

Figure 3. PI-staining in SK-N-AS cells following P4 exposure. SK-N-AS cells were incubated for 1 min with 0.02 mg/mL PI (stock solution 1 mg/mL, 1:50) in medium with gentle shaking and rinsed once with phosphate-buffered saline. Conditioned medium was reapplied and phase contrast and fluorescent pictures were taken immediately with a fluorescence microscope.

Repeated MPA exposure in SK-N-AS cells

To test whether the progestin MPA exerts a cytotoxic effect similar to that of natural P4, SK-N-AS cells were exposed to different concentrations of MPA for 3 and 6 days. Although a significant ($P < 0.05$) increase in cell death was observed after both 3 and 6 d of exposure (Figure 4), the maximum decrease in cell viability attained was only ~20%.

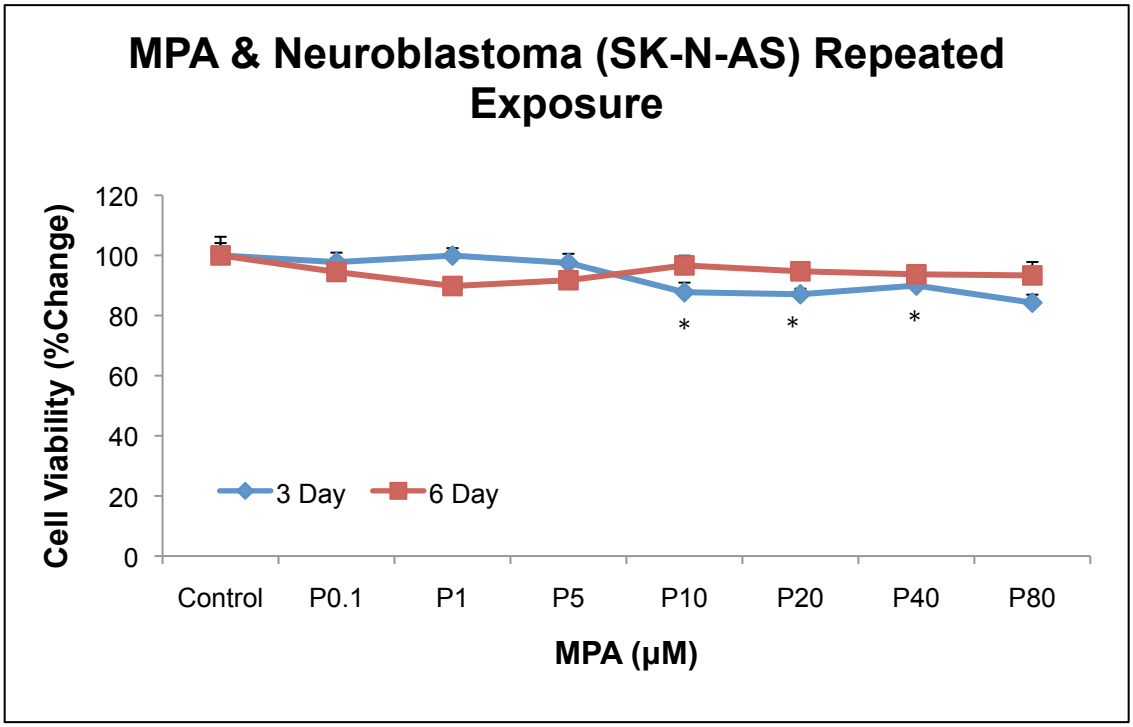

Figure 4. Inhibitory effects of MPA against neuroblastoma growth. Repeated 3 and 6 day MPA exposures at varying doses are compared on the basis of cell viability measured by MTT assay. The stock solution of MPA was prepared in DMSO (final concentration <5 μL/mL medium), then further diluted in culture medium. Culture medium containing MPA was replaced daily. Data are expressed as means ± SE (n=6) from two independent experiments. Significant difference: *P<0.05 compared with vehicle group.

MPA vs. natural P4 exposure in SK-N-AS cells

Repeated 6-day exposures to MPA and P4 in SK-N-AS cells are compared below. Although both P4 and MPA produced a decrease in cell viability, natural P4 clearly exerts a far stronger cytotoxic effect on the tumor cells than MPA. At the highest tested dose (80 µmol/L), natural P4 achieves a further ~60% reduction in cell viability over MPA (Figure 5).

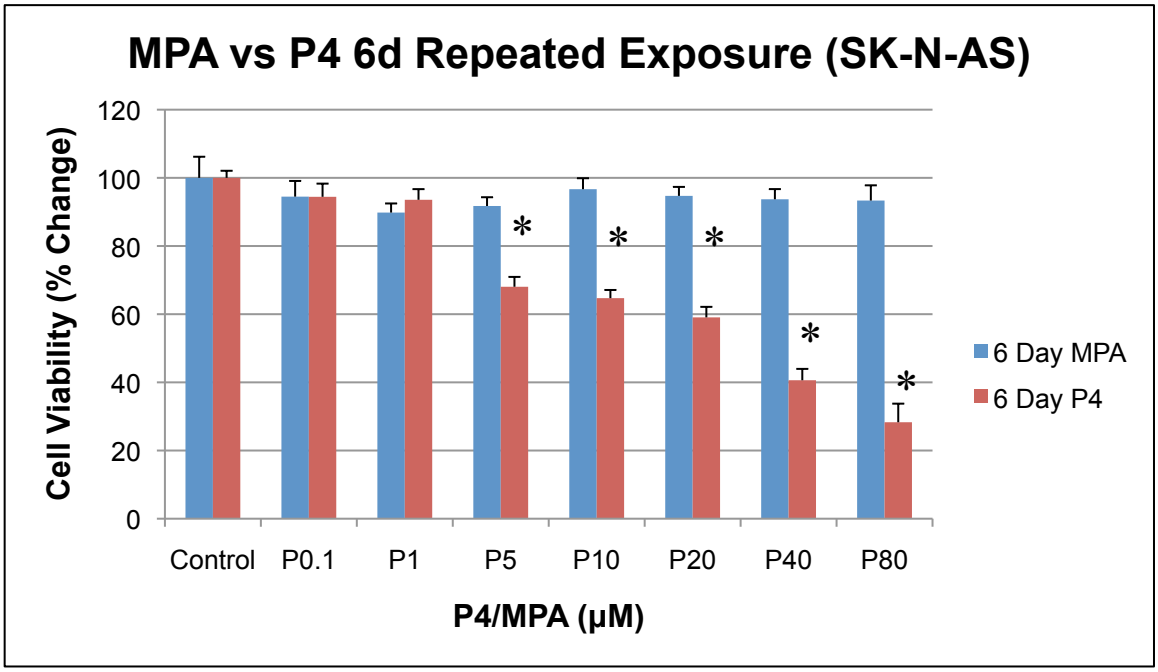

Figure 5. MPA vs P4. Repeated 6-day exposures to MPA and P4 at varying doses are compared on the basis of cell death measured by an MTT assay. Data are expressed as means ± SE. Significant difference: **P*<0.05 compared with vehicle group.

[B]. Effects of P4 and TMZ treatment on GBM cell viability

Single exposure to P4 in U87MG cells

A single 72-hour exposure to P4 at a range of concentrations (0.1, 1, 5, 10, 20, 40 and 80 μmol/L) failed to incite significant ($P < 0.05$) apoptosis at any of the doses tested in human Grade-IV GBM cells (Figure 6).

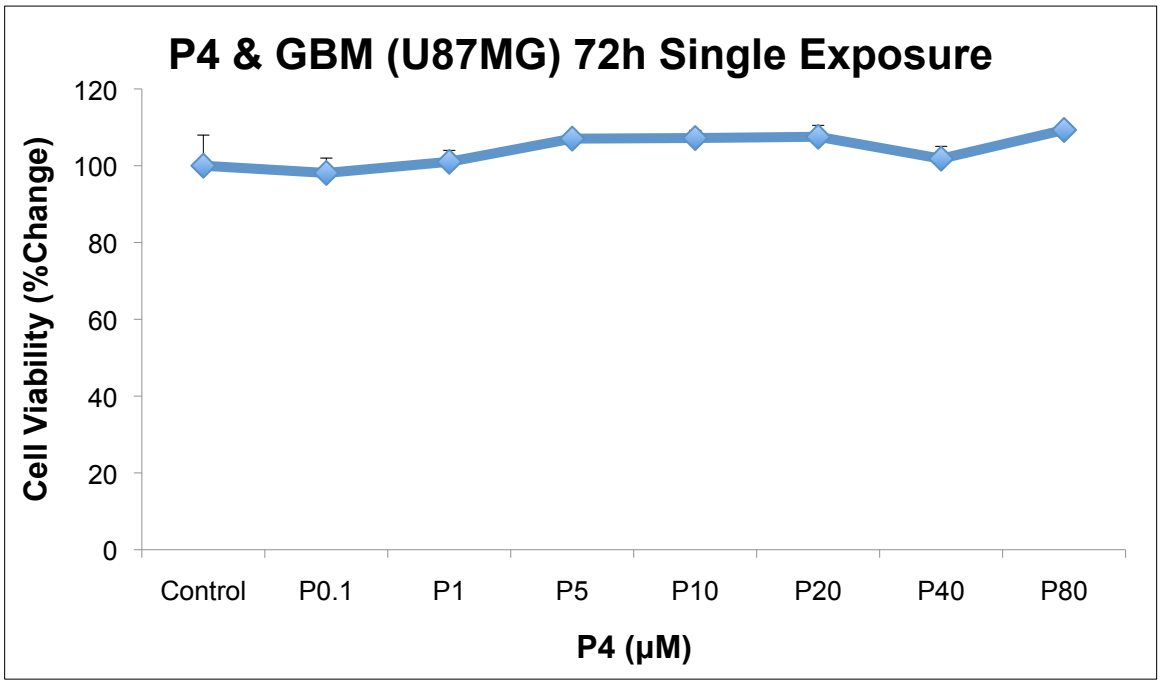

Figure 6. Effect of a single P4 exposure on GBM cells. Cells were exposed to different concentrations of P4 applied once for 72h. Cell death was measured by MTT assay. Data are expressed as means ± SE (n=9) from three independent experiments. Significant difference: *P<0.05 compared with vehicle group.

Repeated P4 Exposure in U87MG Cells

U87MG cells were exposed to different concentrations of P4 for 3 and 6 days. A significant ($P < 0.05$) increase in cell death was observed after both 3 and 6 days following high-dose (20, 40, 80 µmol/L) P4 treatment (Figure 7). Low-dose P4 (0.1 ,1, 5 µmol/L) resulted in a proliferative effect of GBM cells at 6 days.

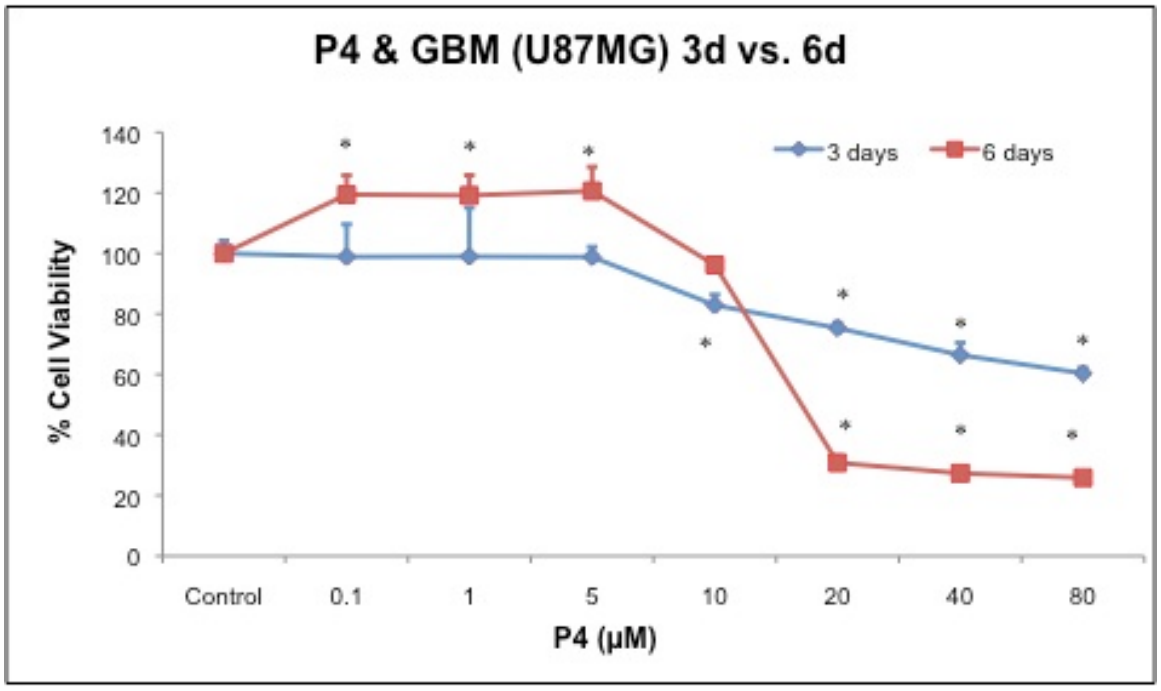

Figure 7. Effect of repeated exposures of P4 on the viability of GBM cells. Cells were exposed to different concentrations of P4 daily for 3 and 6 d. Cell death was measured by MTT assay. Data are expressed as means ± SE (n=12) from three independent experiments. Significant difference: *P<0.05 compared with vehicle group.

Single Exposure to TMZ in U87MG Cells

To test the effects of TMZ alone, the drug was applied once for 3 days. Treatment followed by an MTT assay failed to produce significant ($P < 0.05$) apoptosis at any tested dose (5, 10, 25, 50, 75, 100 and 200 µmol/L) in human GBM cells.

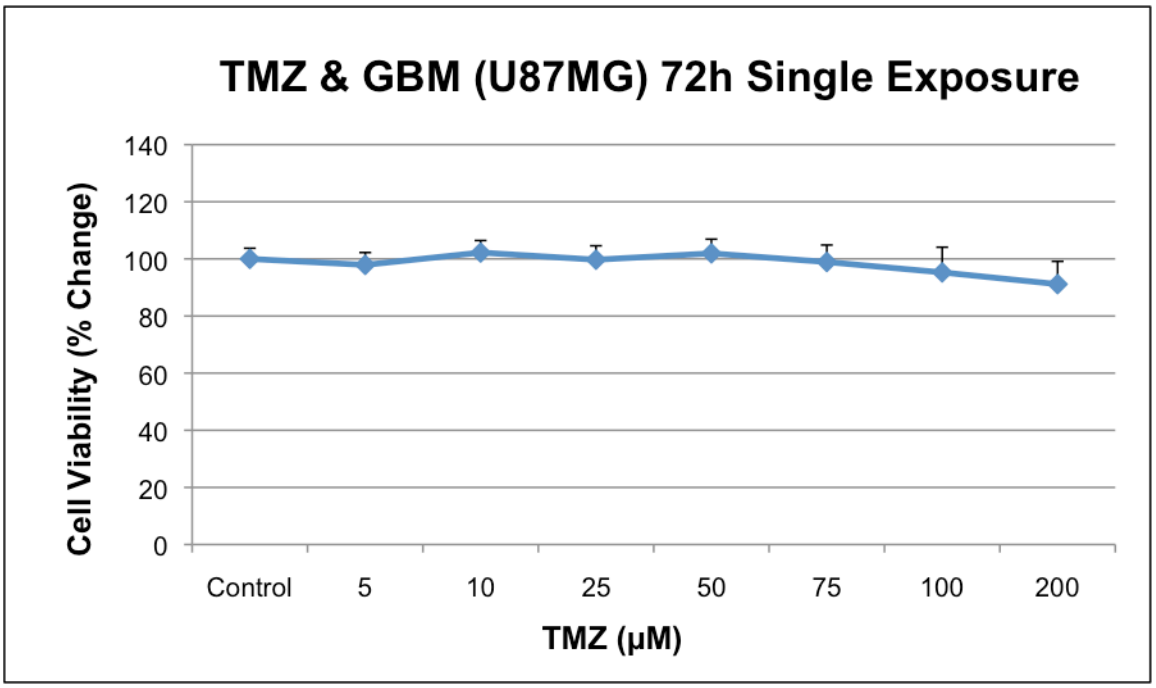

Figure 8. Effect of a single TMZ exposure on GBM cells. Cells were exposed to different concentrations of P4 applied once for 72h. The stock solution of TMZ was prepared in DMSO (final concentration <5 µL/mL medium), then further diluted in culture medium. Cell death was measured by MTT assay. Data are expressed as means ± SE (n=6) from two independent experiments. Significant difference: *P<0.05 compared with vehicle group.

Repeated TMZ Exposure in U87MG Cells

A repeated exposure regimen was performed with TMZ to determine the chemo drugs anti-neoplastic properties *in vitro*. For 3 and 6 days, U87MG cells were exposed to TMZ. Much like P4, a 6-day repeated exposure elicited a greater decrease in cell viability than a 3-day single exposure. Though no significant cell death was observed in the 3-day group, concentrations of TMZ above 25 µmol/L produced significant cell death at 6 days.

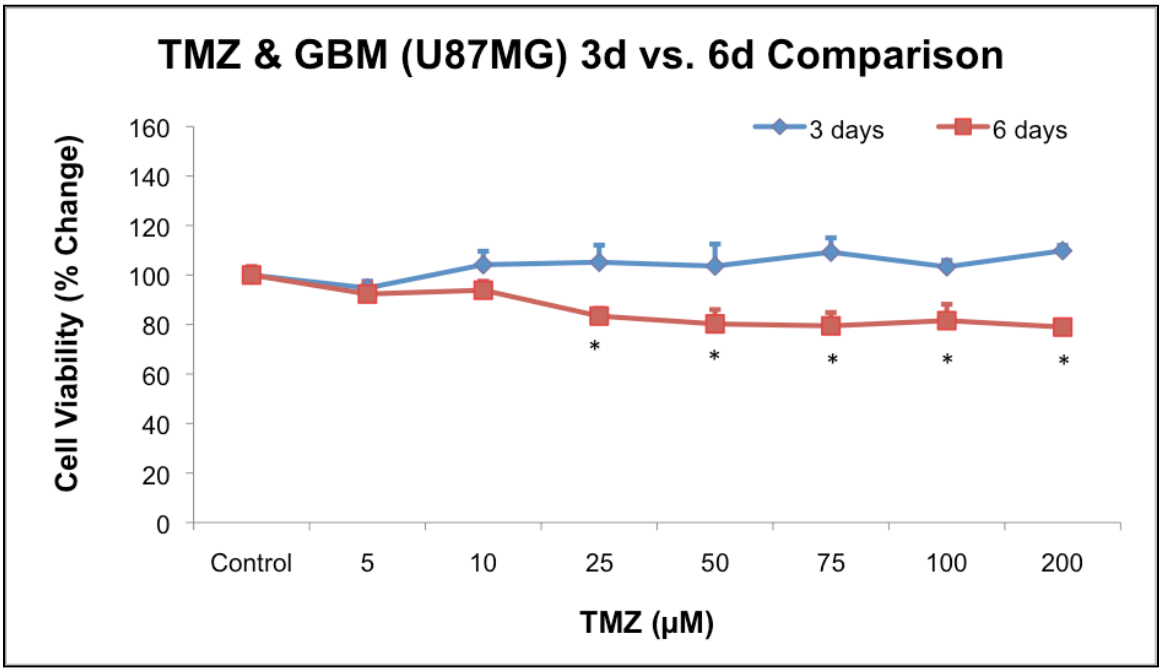

Figure 9. Effect of repeated exposures of TMZ on the viability of GBM cells. Cells were exposed to different concentrations of TMZ daily for 3 and 6 d. Culture medium containing TMZ was replaced daily. Cell death was measured by MTT assay. Data are expressed as means ± SE (n=9) from three independent experiments. Significant difference: *P<0.05 compared with vehicle group.

P4 and TMZ Combined Exposure in U87MG Cells

The chart below (Figure 10) compares the viability of U87MG cells when P4 and TMZ were administered individually and concurrently for 3 and 6 days. In the same trials, P4 and TMZ were also supplemented individually for comparison. At 3- and 6-day groups, we observed the maximum decrease in cell viability at 80 uM concentration of P4. Still, in both 3 and 6 d groups, the combination of P4 and TMZ yielded significantly (P<0.05) more cell death than TMZ alone.

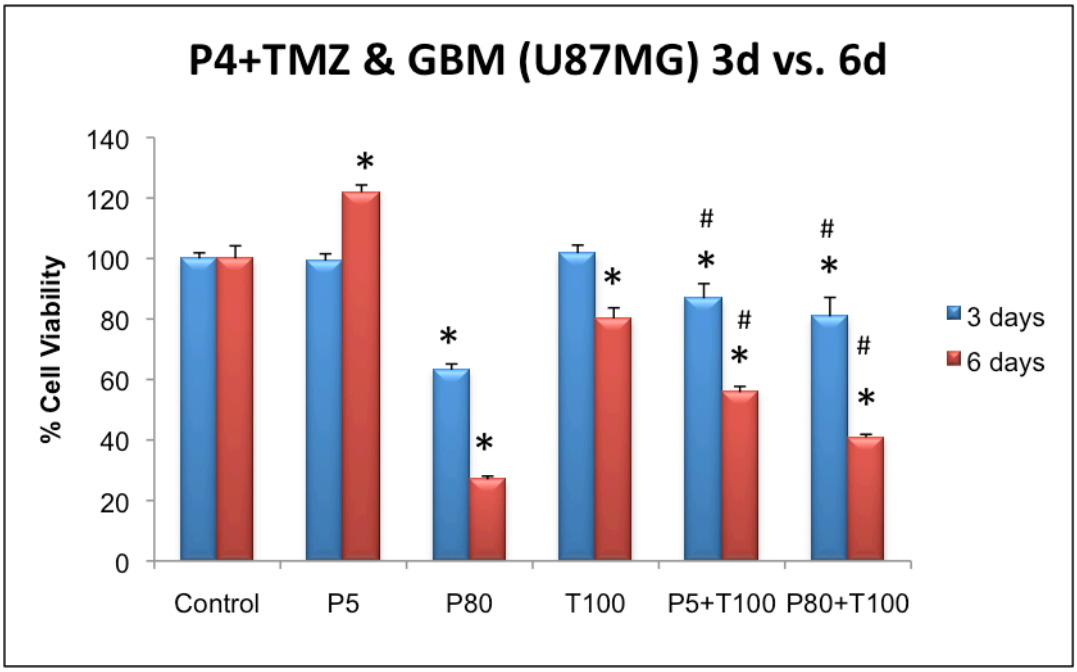

Figure 10. Effect of repeated exposure of P4 and/or TMZ on the viability of U87MG cells. Cells were exposed to different concentrations of P4 and TMZ concurrently for 3 and 6 days. The stock solution of each drug was prepared in DMSO (final concentration <5μL/mL medium). Cell death was measured by MTT assay. Data are expressed as means ± SE (n=9) from three independent experiments. Significant difference: *P<0.05 compared with vehicle group. *P#<0.05 compared with T100 group.

[C] Effects of P4 and TMZ treatment on GBM cell migration

U87MG Cell Migration following P4 and TMZ Exposure

U87MG cell migration was evaluated qualitatively to gauge motility following exposure to P4 and TMZ alone and in combination. In the control group, a substantial number of cells migrated to the injury site at 24 hours compared to 0 hours. P4 (40 and 80 uM) and TMZ (50, 100 uM) markedly decreased GBM cell migration after 24 hours post-wound formation compared to the control group. As shown in Figure 11, high-dose combinations of P4 and TMZ reduced cell movement into the scratch area more effectively than either drug alone.

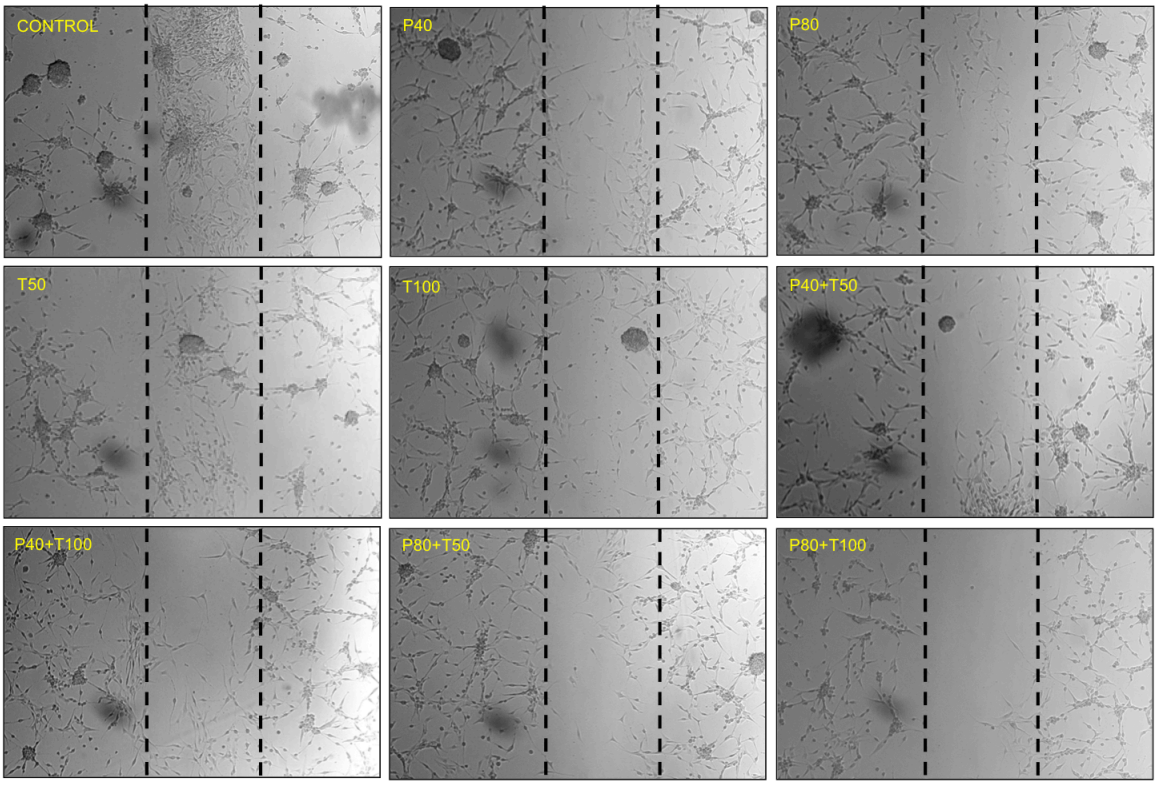

Figure 11: Glioblastoma Cell Migration (Wound Healing Assay). Briefly, GBM cells were grown in multi-well plates and exposed to P4 and/or TMZ at different concentrations. A scratch/wound was formed with a 200µl tip and the cells were kept in an incubator for the next 24 h. Photographs were taken at 0 and 24 h post-wound formation.

[D] Effects of P4 and TMZ treatment on GBM protein expression

P4 modulates cell proliferation in GBM through the EGFR/PI3K/Akt/mTOR pathway

Densitometric analysis of Western blot data revealed a 2-fold decrease in the expression of epidermal growth factor receptor (EGFR) in P80 and P80+T100 groups compared to control (Figure 12A). A 1.25-fold decrease in EGFR was observed in the P5 and T100 groups. Interestingly, the P5+T100 group showed a greater reduction in EGFR (1.4 fold) than T100 alone (1.2 fold). High-dose groups have the largest impact in the reduction of Akt expression as well. In a testament to P4-TMZ synergism We recorded a 5-fold (P80) and 10-fold (P80+T100) decrease compared to vehicle; a far greater reduction of Akt expression than the other groups, which remained at or close to control levels, and (Figure 12B). As shown in figure 12C, the greatest decrease in pAkt expression, 1.4-fold, was recorded in the P80 and P80+T100 groups. Although all experimental groups sustained some reduction in mammalian target of rapamycin (mTOR) expression, again, we recorded the most diminished expression (40%) in P80 and P80+T100 groups (Figure 12D).

Finally, we investigated the expression of PCNA, as an endpoint measure for the EGFR/PI3K/Akt/mTOR pathway. Compared to control, we found that P80 and P80+T100 groups underwent 2-fold and 5-fold declines in PCNA expression, respectively (Figure 12E). It is interesting that despite no recorded change in expression with T100, its combination with P80 enhanced the effect of P80 alone.

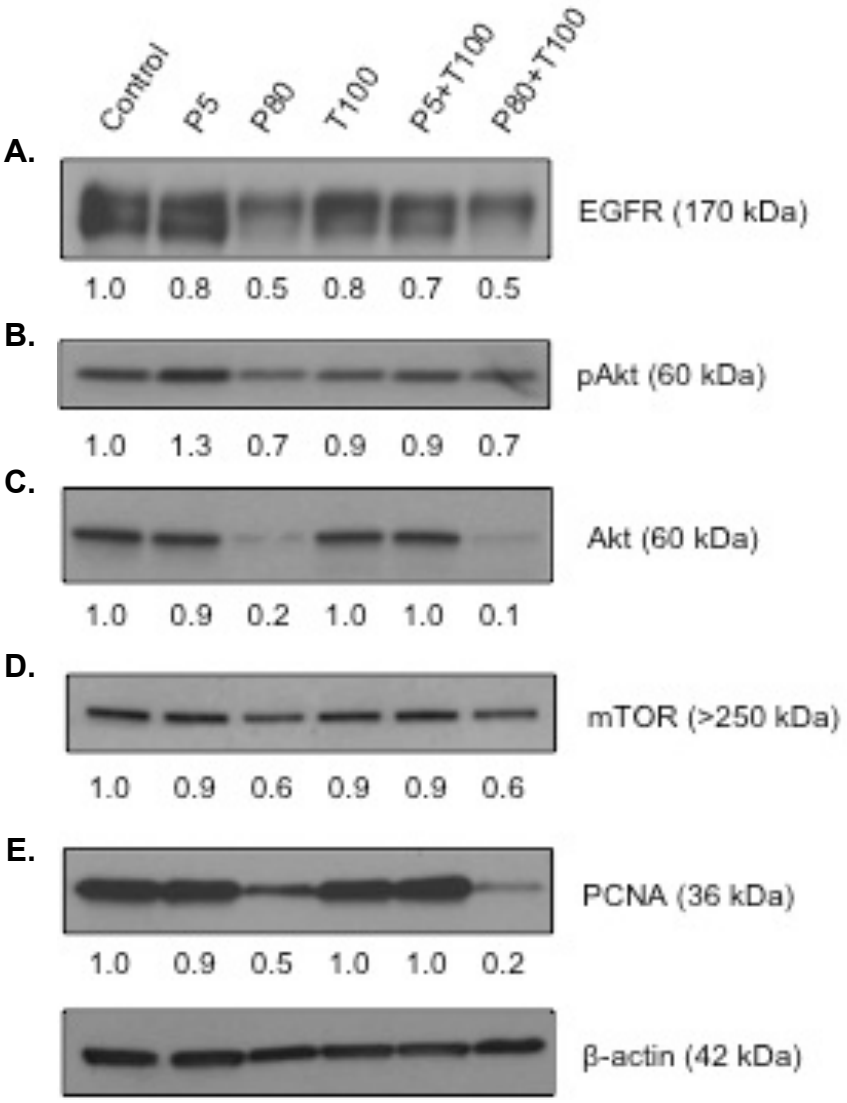

Figure 12: P4 and TMZ modulate proliferation of GBM cells in vitro. Western blot and densitometric analysis of cell proliferation in U87MG cells are shown. Differences are compared with control group. P5: P4 (5 μmol/L); P80: P4 (80 μmol/L); T100: TMZ (100 μmol/L).

DISCUSSION

Taken together, our findings strongly suggest that, at high doses, P4 inhibits the growth of neuroblastoma and GBM *in vitro*, a response not attained by its synthetic counterpart, MPA. Additionally, using the more aggressive U87MG GBM cell line we demonstrate a significant reduction in cell viability and cellular migration achieved through the synergistic combination of P4 and TMZ, the current standard-of-care chemotherapeutic.

As the global burden of cancer continues to grow, researchers and clinicians have sought to develop advanced and targeted treatments with the hopes of reduced morbidity and robust survival. Though leaps in treatment efficacy have been made, there is still much room for improvement. For example, though the 5-year survival rate for patients with neuroblastoma has increased from 52% to 74% between years 1975 and 2005 [4], survival for individuals with more high-risk forms remains below 50% [25]. Meanwhile, even more aggressive cancers such as GMB have had relatively static overall mortality rates.

One of the foremost issues with current chemotherapeutic treatments is their cytotoxic effect not only on cancer cells, but on healthy tissue as well. An agent that maintains the viability of healthy cells and selectively kills cancer cells could be an important advance for chemotherapy development. It has been shown that P4 does just that. When healthy primary cortical neurons and fibroblasts were exposed to various doses of P4, no cell death was observed at any concentration [22]. Our findings in this study, with both neurogenic cancers, can be taken to support the notion that P4 has some degree of specificity in

inducing the death of tumor cells. Though an exact mechanism is yet to be determined, there is further evidence of P4's pleiotropic nature. Atif *et. al*. reported that P4 at high concentrations (20, 40 and 80 μmol/L) prevented the death of primary cortical neurons when exposed to glutamate toxicity, with 20 μmol/L affording the greatest protection [26]. In the present study, we report the cytotoxic effects of P4 on both neuroblastoma and GBM at the same high concentrations (20, 40 and 80 μmol/L) as evidenced by PI staining and cell viability studies utilizing an MTT assay.

Some recent studies support and some contradict our finding that P4 exerts toxic effects on tumor cells [27,28,29]. It is imperative, however, to consider the form of progestogen used in the many studies that compose the knowledgebase. Both natural P4 and its synthetic analogs, called progestins, fall under the umbrella term progestogen due to their progestational effects in the uterus. While natural P4 is synthesized from either soybeans or wild yams, it is in fact bio-identical to the hormone the human body produces, indicating identical molecular structures. Contrastingly, though progestins may have fundamentally similar structures, they are not molecularly identical to P4 and were created in an effort to enhance the half-life of P4 and for patent purposes. For these reasons, different progestogens used can elicit drastically different responses in a biological system. The progestin MPA has been reported to exert substantively different metabolic effects and have different target genes from those of P4 [14]. Several clinical trials found that, in hormone replacement therapy, the combination of estrogen with a progestin such as MPA increased the risk of

breast cancer [30]. Two cohort studies followed over mean durations of 7 and 9 years showed no increase in breast cancer risk when estrogen was combined with natural P4, but there was an additional risk when synthetic progestins were used [27,28]. Although P4 and MPA have similar structures, MPA differs in its two added side groups (Figure 13), which could explain their differing effects.

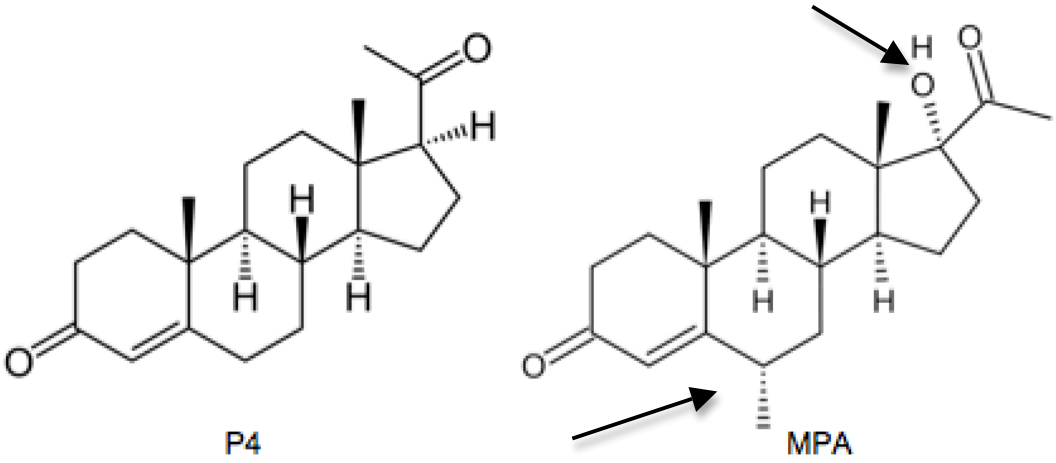

Figure 13: Natural Progesterone vs MPA. Here the structures of P4 and MPA are compared [46,47]. Although much of the structures are analogous, notice the changes in side-chains designated by the arrows.

As with any hormone or drug, P4's effects are greatly influenced by the dose administered. In the course of other projects in our lab, we discovered that a high dose of P4 was necessary to achieve toxicity in tumor cells. Conversely, through *in vitro* dose response studies, we reported GBM tumor cell proliferation with low-dose P4 treatments. The critical importance of P4 dosing is made clear through an *in vivo* study performed by Benakanakere *et al.* [29] in which they report that both P4 and MPA accelerate 7,12-dimethylbenz(*a*) anthracene (DMBA)-initiated mammary tumors in rats. They demonstrate that P4 treatment 4 weeks following DMBA treatment leads to an increased incidence of mammary

tumors but has no effect on latency period, while MPA treatment significantly increased tumor incidence and a reduced latency period. We hypothesize that the elevated tumor incidence associated with P4 treatment is attributable to the low dose (10 mg/60-day release 0.166 mg/day) utilized by Benakanakere et al. [29]. A similar *in vivo* study by Atif *et. al.* used much higher P4 dosing (~2.2–2.5 mg/day) which resulted in a ~50% inhibition of tumor growth [22]. Although the present study applies a different experimental design, the results of both Benakanakere *et al.* and Atif *et. al.* support our *in vitro* findings which show a proliferative effect with very low concentrations of P4 (1,5 μmol/L) and cytotoxic effects with high concentrations (20,40,80 μmol/L) in neurogenic tumor cells following 6-day repeated exposures.

Having established effective dosing concentrations and observed the efficacy of repeated high-dose P4 treatments in SK-N-AS neuroblastoma cells, we began to test P4 on more aggressive, grade IV, U87MG human GBM cells. P4 could prove especially relevant in the treatment of GBM, an intracranial tumor, as it freely passes through the BBB. In the current experiment, we found a potent inhibitory effect of high-dose P4 as it significantly reduced GBM cell viability. A leading problem with GBM is recurrence, which happens in almost 100% of cases [7] and results in even more aggressive tumor cells. Consequently, a primary therapeutic goal is to maximize tumor cell apoptosis, a goal that is often enhanced through combination chemotherapy.

TMZ is the standard of care chemotherapeutic used to combat GBM. The alkylating agent effectively crosses the BBB with less severe adverse effects

than many other chemo drugs [31]. However, it is common for tumors to become resistant to TMZ by, for example, raising O^6-alkylguanine-DNA alkyl-transferase (AGT) levels or causing mismatch repair (MMR) deficiencies, both of which impede the intended clinical response in cancer patients. Furthermore, many chemo agents have mutagenic properties, which facilitate the emergence of potentially resistant tumor cell clones [32]. To abrogate this resistance, scientists in 1965 found that using different drugs with diverse mechanisms of action concurrently would make it more difficult for cancer to develop a resistance to the combination [33].

In the present *in vitro* study, we investigated the effects of TMZ alone and in combination with P4 on GBM cell viability in an effort to attenuate resistance and increase the efficacy of TMZ. First, we performed dose response studies with TMZ alone to assess optimal dose concentrations. Then, having established the optimal levels of P4 and TMZ in isolation, we tested concurrent dosing of GBM cells with numerous combinations of P4 and TMZ. The most effective combinations are reported in this study. We found that the potential combinatorial drug, P4, does in fact enhance apoptosis of GBM cells, producing a synergistic response when combined with TMZ as opposed to TMZ alone. We showed that cytotoxicity following 3- and 6-day treatment was significantly greater when TMZ was combined with low-dose (5 μmol/L) and high-dose (80 μmol/L) P4 compared to TMZ alone. The model adapted and used to test concurrent treatments in this study is derived from a comparative study in which TMZ was combined with the naturally occurring polyphenolic compound resveratrol in an effort to treat grade

IV GBM (SGH44). Though the cell line is genetically different in our study, the researchers found a similar and significant synergistic effect in reduction of cell viability with concurrent treatment versus TMZ alone [34]. The ability to resist cell death is among the six hallmarks of cancer outlined by Hanahan *et. al.* [35]. Our study has shown that P4 and TMZ treatments can work in concert to obstruct tumor cells from evading apoptosis.

Human tumor development is a very complex process. The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. These hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [35]. To explore the effects of concurrent P4 and TMZ treatment on another cancer hallmark, cell migration, we performed wound-healing experiments to examine the effect of P4 and TMZ alone or in combination on U87MG cell migration. Our findings show both TMZ (50,100 μmol/L) and P4 (40, 80 μmol/L) individually reduce migration into the scratch area when compared to control. However, cell movement was most hindered when high doses of both P4 and TMZ (P80+T100) were applied concurrently. Collectively, our *in vitro* data shows that the combination of the two drugs work in concert to improve the reduction of cell viability and migration more effectively than TMZ alone.

To better understand the mechanisms that may underlie the data collected, we focused our protein expression studies on the EGFR/PI3K/Akt/mTOR pathway (Figure 14), a key signaling pathway in the development of GBM whose activity is also a marker for cell proliferation [36].

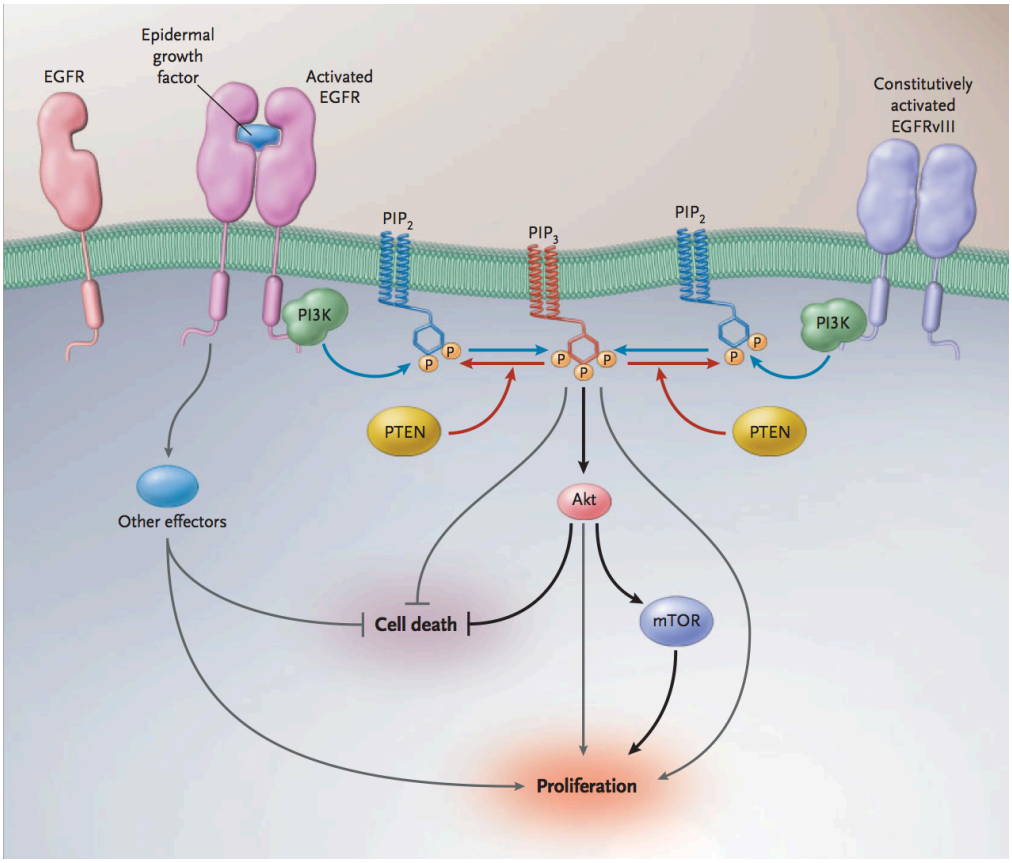

Figure 14: The EGFR/PI3K/Akt/mTOR Signaling Pathway in GBM. Upon EGFR activation, PI3K is recruited to the cell membrane. PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to the second-messenger molecule PIP3 (blue arrows). This second messenger then activates downstream the molecules Akt and the mammalian target of rapamycin (mTOR), which help induce cellular proliferation and block apoptosis. PTEN terminates the PIP3 signal (red arrows) [48].

Recall that sustaining proliferative signaling is another among the hallmarks of cancer. EGFR amplification occurs in ~40% of glioblastomas with overexpression occurring in over 60% of cases [37,38]. Mutation and

overexpression of EGFR have been linked to the development of more aggressive malignant phenotypes leading to increased resistance to treatment and poorer clinical outcomes [39,40]. In our study we found that all treated groups had reduced EGFR expression. However, the P80 and P80+T100 groups attained the largest reduction, measured at two-fold. It is interesting to note that TMZ supplemented with P4 (5 μM) was more effective at reducing EGFR expression than TMZ alone: 20% versus 30% respectively.

Upon EGFR activation induced by growth factors (epidermal growth factor, transforming growth factor-α) binding to its extracellular domain, PI3K is recruited to the cell membrane. Once activated, PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) via phosphorylation of phosphotatidylinositol-4,5-bisphosphate (PtdIns (4,5)P2) [41]. PI3K function is antagonized by phosphatase and tensin homolog (PTEN), an important tumor suppressor, which dephosphorylates PtdIns (3,4,5)P3 to PtdIns(4,5)P2.

Initiated by the presence of PtdIns (3,4,5)P3, Akt translocates to the plasma membrane as it is activated, leading to a conformational change in Akt. Subsequently, Akt is phosphorylated at three regulatory sites; we chose to study Ser^{437} [42]. Once activated, Akt phosphorylates multiple downstream targets involved in important cellular processes including apoptosis, cell proliferation, metabolilsm, and cell growth. Additionally, Akt promotes cell survival by promoting the induction of cell survival proteins and blocking the function of pro-apoptotic proteins [43]. Western data reveals a 5-fold decrease in Akt expression when cells were treated with P80. Although no effect was recorded with TMZ

alone, Akt expression declined most, 10-fold, when TMZ was supplemented with P80, indicating a synergistic combination. Additionally, we recorded a 1.4 fold decrease in pAkt expression in the P80 and P80+T100 groups.

A major downstream effector of Akt is mTOR (mammalian target of rapamycin), which regulates cell growth through the coordination of growth factor and nutrient signaling [44]. Our experiments reveal a 10% decrease in mTOR expression when U87MG was treated with P5, T100 or P5+T100, and a 40% expression decline when dosed with P80 or T100+P80. The Western analysis above consistently shows that P80 and P80+T100 groups are most effective, in some cases more so in combination, at reducing expression along the EGFR/PI3K/AKT/mTOR pathway responsible for cell proliferation signaling.

Next, we examined the expression of PCNA, a protein that is often used for grading different neoplasms. This protein is of interest especially in conjunction with the EGFR/PI3K/Akt/mTOR pathway because PCNA levels serve as endpoint measures for cell proliferation and cell-cycle progression. PCNA is synthesized early in the G_1 and S phases of the cell cycle as it forms a ring around DNA to facilitate and control DNA replication [45]. Additionally, PCNA has been found to play a role in chromatin structure maintenance and chromosome segregation, all vital elements of the cell cycle [45]. P4 at a 80 μM concentration resulted a 2-fold reduction in PCNA expression, indicating a higher rate of U87MG cell cycle arrest. Furthermore, the addition of P80 to T100 enhanced the decline of PCNA expression 5-fold, a synergism not attained with TMZ plus low dose P4 and an effect far more potent than TMZ alone.

Taken together, our data illustrates the potential of enhancing the effects of TMZ with the addition of P4. In spite of the promising reductions in cell migration and cell viability, findings supported by favorable changes in key tumorgenic protein expression, many areas are in need of further investigation. Though *in vivo* research has shown that P4 can inhibit neuroblastoma growth by ~50% [22] and that a combination of TMZ and resveratrol can be used to reduce GBM tumor size more effectively than TMZ alone [34], the experiments in this work are entirely *in vitro*. We cannot account for the complexities and interactions that take place in a biological system when TMZ and P4 are combined. Also difficult to overcome is tumor heterogeneity. The many genetically distinct forms of the same cancer make it especially difficult to find a comprehensive treatment. Here we experimented on just one of the innumerable neuroblastoma and GBM cell lines. Although similar responses to P4 and TMZ have been recorded in other neurogenic cell lines [22,34], a more comprehensive study of concurrent drug administration in numerous cell lines is needed to fully understand the extent of P4 plus TMZ application. Also, while cell migration is reduced most with concurrent P4 and TMZ administration, P4 alone is more effective at reducing cell viability. Thus, the question of whether the drugs should be used in concert or individually needs further study. A more sustained administration of the drugs *in vitro* and *in vivo* could elucidate the costs and benefits of treatment regimens in the long term.

There are still many key issues that remain to be investigated concerning P4 and TMZ combinations. Although we have shown favorable effects on three

cancer hallmarks, elucidation of the drugs' effects on the other three hallmarks - evading growth suppressors, enabling replicative immortality, and inducing angiogenesis - could provide additional support for the validity of concurrent treatment. Eventually this line of research will need to be extended to an *in vivo* intracranial model in order to mimic the tumor microenvironment as closely as possible. There is much room for this work to grow in different directions as well. P4 has reliably proven to be safe, even at very high doses. Its application for TBI, backed by two independent Phase III trials that are nearing completion, could be extended to the field of oncology. P4's neuroprotective effects have the potential to defend healthy cells during radio and chemotherapy. If P4 is effective, clinicians may be able to treat the cancer more aggressively in order to reduce morbidity and minimize the possibility of recurrence. It would also be interesting to begin experiments that combine P4 with a particular cancer's standard of care chemotherapeutic to determine whether similar synergistic effects could be achieved. Although this is a first step in this direction, much more research is needed before going to clinical trial. Therefore we should continue to investigate P4's interactions along with varying levels of radiation, surgery or chemotherapy to improve functional outcomes and patient survival.

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DISCLOSURES

1) A US patent (# US 8,435,972 B2) was issued to Fahim Atif and Donald G. Stein on May 7, 2013 for the use of P4 and compositions related thereto for the treatment of neurogenic tumors specially neuroblastoma and glioblastoma.

(2) DG Stein receives royalties from products of BHR Pharmaceuticals Ltd related to the use of progesterone in the treatment of TBI and stroke, and may also receive research funding from BHR Pharmaceuticals, which is developing products related to this research. Some of these royalties are placed into a laboratory account used to support ongoing research. This amounts to 1.5% of royalties. 7.5% went to Dr. Stein for discretionary use. Over 60% of royalties goes to the University senior administration and various Deans, and another small share to the Chair of the Department of Emergency Medicine. In addition, Stein serves as an occasional consultant to BHR Pharmaceuticals and receives compensation for these services. The terms of this arrangement have been reviewed and approved by Emory University, which will continue to receive the largest share of fees and royalties in accordance with its conflict of interest policies.