Failure of Inositol Trispyrophosphate to Enhance Highly Effective Radiotherapy of GL261 Glioblastoma in Mice

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Abstract. *Background/Aim: Inositol trispyrophosphate (ITPP), reported to cure hepatomas in a preclinical rat model and to have beneficial effects in several other solid tumor models, is currently in clinical trial for liver cancer. We investigated whether aggressive glioblastomas could be effectively treated with ITPP alone or in combination with radiation therapy (RT). Materials and Methods: C57Bl/6 mice were intracranially injected with syngeneic GL261 glioblastoma cells and treated with hypofractionated radiation (5 Gy × 3), ITPP, or both. Tumors were followed by imaging, and mice sacrificed due to morbidity or at 90 days, with microscopic examination of brain sections. Results: RT alone significantly prolonged survival, whereas ITPP alone did not. Surprisingly, ITPP appeared to reduce the effectiveness of RT when added in combination. Conclusion: ITPP was ineffective as monotherapy for glioblastoma and appeared to interfere with the beneficial impact of RT.*

Glioblastoma multiforme (GBM) is among the most difficult to treat malignancies. In part, this is due to the essential function of the brain, the difficulties of treating a tumor within a confined space, and the problems of drug penetration through the blood–brain barrier. Additionally, it has long been noted that the relative hypoxia within brain tumors may interfere with the efficacy of radiation therapy (RT) and various antitumor drugs, while promoting the survival of cancer stem cells (1-5). Disturbingly, radiation has been implicated in triggering new mechanisms of GBM invasiveness involving activation of Rho kinase (6). Nevertheless, radiation remains a mainstay of GBM treatment, providing up to several months of delayed progression (7-10).

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We reasoned that reversal of tumor hypoxia would, in theory, be highly beneficial, slowing or reversing hypoxia-inducible factor (HIF)-mediated changes that promote tumor progression, and enhancing the response to drugs and RT. To this end, we employed a novel candidate drug, inositol trispyrophosphate (ITPP). This small molecule is actively transported by the erythrocyte membrane 3 anion transporter protein into red blood cells, where it binds to hemoglobin allosterically, with higher affinity for deoxyhemoglobin. The reported result is a 'right shift' of the oxygen-hemoglobin dissociation curve, such that more oxygen is released from hemoglobin at lower ambient oxygen concentrations. Thus, the hypoxia of tumor regions should be partially reversed. ITPP has proven highly active in several pre-clinical models of solid tumors, with evidence of reduced tumor region hypoxia and reduced expression of hypoxia-inducible factor (HIF) and HIF-regulated pathways (11-15). Phosphatase and tensin homolog (PTEN) levels were also shown to be elevated after ITPP treatment (11), and growth of glioma cells in chorionic allantoic membrane cultures was inhibited (14). ITPP is currently in clinical trials at the University of Zurich, for liver and pancreatic cancer. We hypothesized that ITPP would also be beneficial in combination with RT of gliomas.

The GL261 cell line is a C57BL/6-derived aggressive astrocytoma that has been used in many studies of orthotopic syngeneic modeling of glioblastoma. It is moderately radiosensitive, and can be immunogenic under some conditions (16-20). In particular, Zeng *et al.* showed that non-curative irradiation can be combined with the immune checkpoint inhibitor, antibody to programmed death receptor (PD1), to generate highly protective immunity against established GL261 tumors (20). Since physiological normoxia, like checkpoint inhibitors, should support effector immunity and reduce hypoxia-associated tumor tolerance, ITPP and RT were assessed separately, and in combination, as treatments for established GL261 tumors in the C57BL/6 orthotopic syngeneic model.

Materials and Methods

Mice. C57BL6 female mice were purchased from Charles River Laboratories (Wilmington, MA, USA), housed in the Beaumont Radiation Animal Facility (Royal Oak, MI, USA), and used at 9-11

weeks, at which time they weighed 25-30 g. This work was performed under Oakland University IACUC approval # AL-14-05.

Tumor injection. Twenty female C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were implanted intracranially with 1×10^5 GL261 cells (National Cancer Institute Repository, Frederick, MD, USA) in 2 μl of sterile phosphate-buffered saline on day 0. Mice were 14 weeks of age at the start of experiment and weighed ~22 g. The implant site was 1 mm posterior to the bregma and 1 mm lateral of the midline suture on the left side of the brain. Small alterations in position were made to avoid obvious vasculature when possible. For implantation, a 26G Hamilton syringe was inserted 4 mm into the brain and then withdrawn to 3.5 mm for injection, which was performed over several minutes. The cranial bore-hole was sealed with bone wax following withdrawal of the needle and the incision closed with surgical glue.

Magnetic resonance imaging (MRI). Seven days post implantation, and at weekly intervals thereafter, animals received diluted Multihance (Bracco Diagnostics, Inc., Cranbury, NJ, USA) *i.v.* and underwent contrast-enhanced MRI under anesthesia. Fast spin-echo T1- and T2-weighted images were acquired at 0 and 5 minutes post-contrast injection, and tumor volumes were determined by delineating areas of contrast enhancement.

Group distribution of animals. In order to ensure statistically similar tumor burdens among all four groups of mice, animals were ranked for tumor volume, and then assigned to one of four treatment groups (placebo, RT, ITPP, or RT plus ITPP) using an algorithm that minimized differences among means and SEMs among groups. One-week mean tumor volumes among groups post-assignment ranged from 5.6 to 6.6 mm^3, and were not statistically different among groups.

Treatments, monitoring, and statistical analysis. On the eighth day post-tumor implantation, ITPP treatments were begun, using an *i.p.* dose of 1 g/kg every 4 days five times. This dose is within the range that has proven effective against liver and colon tumors in mice and rats (11-14). RT was initiated in the RT and RT plus ITPP groups one day later (day 9). We used a hypofractionated dose of 5 Gy every 4 days three times, rather than a single dose like Zeng *et al.* (19), or the more typical daily or every-other-day fractionated doses used in tumor RT models, including models where non-metastasizing GL261 tumors are established and treated in the flanks of mice (21). Radiation was delivered to the whole head using a Faxitron cabinet irradiator, at a rate of 0.25 cGy/min to body-shielded mice. A total of 15 Gy was delivered in three 5 Gy/day doses spaced 4 days apart (*i.e.* over an eight-day window from day 9-17 post-tumor injection).

In addition to the MRI, mice were monitored daily for weight and signs of neurological impairment, including: lethargy, poor grooming, asymmetric gait, hunched posture, head position asymmetry, any abnormal movements. Mice exhibiting more than 20% weight loss, or progression of any of the above signs for more than 2-3 days were sacrificed. Mice surviving without signs for more than 90 days were sacrificed by cervical dislocation after xylazine/ketamine overdose. Survival curves were analyzed by the Gehan-Breslow-Wilcoxon method.

Sacrifice and brain histopathology. Upon sacrifice, brain tissue was collected from all animals and fixed in paraformaldehyde for further histological studies. Tissue samples were then blocked and hematoxylin and eosin (H&E) slides stained for each sample. Images were obtained at ×10 and ×40 magnification and examined quantitatively for regions of normal brain, tumor, and hemorrhage.

Results

Figure 1 summarizes the group survival curves. Control mice receiving GL261 cells died or were sacrificed due to neurological signs between 19 and 27 days post-tumor implant (mean survival=26.5 days). Disappointingly, mice receiving ITPP treatment, starting on day 8 post-tumor implantation, showed no improvement in survival, with animals dying between days 21 and 25 (mean=23 days). Surprisingly, strong beneficial results were seen for the group treated with RT only, which had the longest mean survival time of 64 days. Two animals survived the full 91-day observation period, with stabilization or reduction in tumor size by MRI after initial growth. One animal sacrificed at day 49 due to weight loss, pallor, hunched posture and poor ambulation, showed no evidence of residual tumor on H&E histopathology examination of the brain (possibly dying from other causes after clearing tumor burden). The two mice sacrificed in good health at 91 days had no histopathological evidence of tumor on H&E staining (Figure 2, upper right).

When added to RT, ITPP failed to confer any survival benefit. Indeed, the mean survival time for the group treated with RT plus ITPP (46 days) was borderline significantly less than that of the group treated with RT only (*p*=0.06). One mouse receiving the combined RT plus ITPP therapy survived the full 91-day observation period and had no histopathological evidence of tumor in brain sections.

Tumor volumes assessed by MRI showed significant effects of RT, with lower volumes at different post-injection time points. As expected, mean tumor volume growth during the first 6-8 weeks post-implant corresponded inversely with group survival rates (Figure 3). Interestingly, ITPP-treated animals, as a group, were deemed terminal at lower tumor volumes (94 mm^3) than control animals receiving saline (136 mm^3). This was also true for tumor volumes in RT-treated mice also receiving ITPP (87 mm^3) *vs.* those receiving only RT (177 mm^3) sacrificed for morbidity. The lower tumor volumes by MRI at death were generally consistent with smaller areas of tumor-involved brain upon histopathological examination. It is possible that these low tumor volume deaths reflect microscopic satellite areas of invasive spread not detected with MRI or histopathology.

Discussion

Two key points emerge from this study. Firstly, contrary to expectations, ITPP was not beneficial with respect to survival, and may have been detrimental when added to RT.
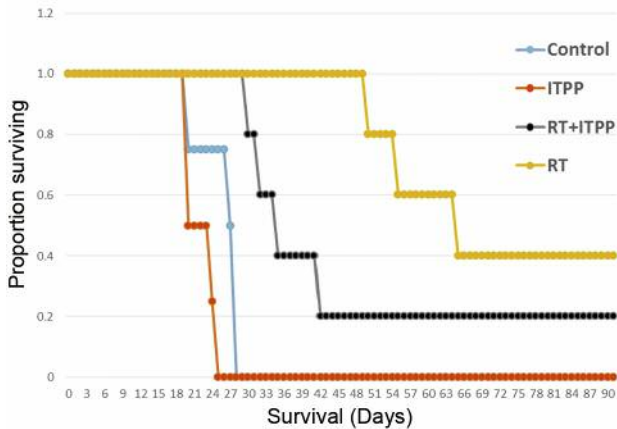
Figure 1. Survival curves for mice orthotopically injected with GL261 cells. The median survival compared by the Grehan-Breslow-Wilcoxon test) of control mice (26.5 days) was not significantly different from those treated with inositol trispyrophosphate (ITPP, 23 days). Radiotherapy (RT) significantly extended mean survival (64 days, p<0.005). When ITPP treatment was added to RT, the mean survival of 46 days was still significantly extended vs. controls (p<0.006). However, there was a trend toward reduced mean survival in ITPP + RT vs. RT only (p=0.06), indicating a possible detrimental impact of ITPP on the benefit of RT.

Figure 2. R*epresentative hematoxylin & eosin stained coronal slices of mouse brains from the four treatment groups were examined and photographed (original magnification, ×10). Regions of normal brain tissue (pink), tumor plus infiltrating leukocytes (purple), and hemorrhage (red) are visible. RT: Radiation therapy; ITPP: inositol trispyrophosphate.*

Secondly, a total of 15 Gy given in three equal 5 Gy doses at 4-day intervals, starting 9 days after tumor injection, was highly protective, with an overall 'cure' rate of 30% among 10 mice receiving RT as a component of therapy (with or without ITIPP). In addition, one mouse receiving RT alone died without MRI or histopathological evidence of tumor. If this animal cleared its tumor, as histopathology suggests, then 40% of mice had no residual tumor following RT. This degree of tumor regression has not previously been described for RT of orthotopic GL261 tumors.

Because reported success in augmenting RT of GL261 cells has involved potentiation of immunity (*e.g.* 16, 19, 20), the immune response may also have been crucial in our long-surviving irradiated mice, as well. We hypothesize that the hypofractionation regimen used was sufficient to cause cell death and generate immunogenic debris, while sparing sufficient numbers of immune-responsive cells. More highly fractionated RT regimens, such as daily dosing, may expose a greater proportion of immune cells to apoptosis-inducing radiation, thereby suppressing the potential benefits of an immune response (22). At the other extreme, in the study of Zeng *et al*., a single 10-Gy dose of radiation modestly delayed orthotopic GL261 tumors but did not cure any mice (20). Importantly, sham-treated control animals in that study died within the same 19- to 30-day window as our saline-treated controls. However, it is worth noting that the
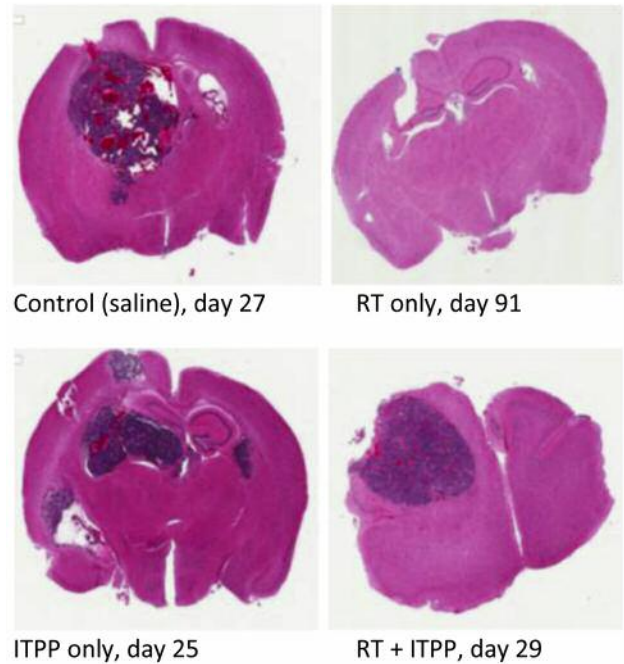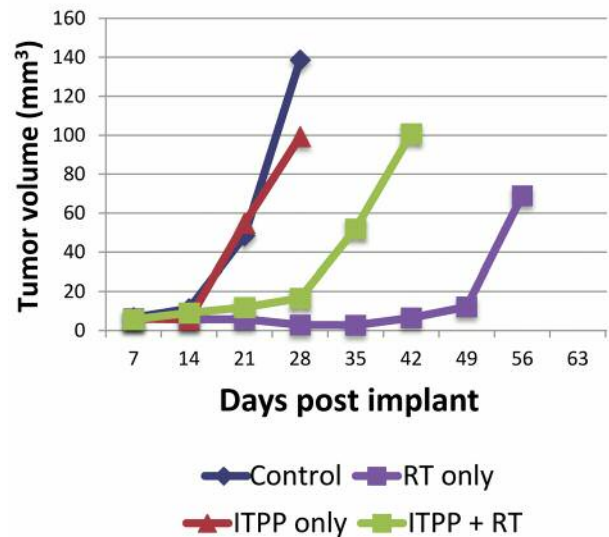

Figure 3. *Average tumor volume as measured by contrast magnetic resonance imaging for treatment groups. Weekly imaging after GL261 orthotopic injections revealed rapid growth among control mice receiving saline, and mice receiving only inositol trispyrophosphate (ITPP). Mice treated with ITPP plus radiotherapy (RT) exhibited an approximately 2-week delay in rapid tumor growth, while animals receivinig RT only had about 5 additional weeks of relatively slow growth compared to controls. Data from surviving tumor-free mice is not shown.*

GL261 tumor cells used in the study of Zeng *et al.* were transfected with luciferase, a likely immunogen (23). Thus, an anti-luciferase response may have contributed to the dramatic results seen in mice treated with anti-PD1 plus RT. It will be important to formally demonstrate an immunological component in the phenomenon of RT-induced cures in our experiments.

The failure of ITPP to improve outcomes, and the suggestion of actual acceleration of tumor pathology, are disappointing but also intriguing findings. Mice tended to die sooner, and with smaller tumors, when they had received ITPP. After performing these experiments, we became aware of the study of Förnvik *et al.* showing no benefit of ITPP monotherapy in the treatment of orthotopic syngeneic rat RG2 glioblastoma, and a possible negative survival impact of ITPP on rats injected with RG2 subcutaneously (24). Although RT was not used in that study, the results support the conclusion that ITPP is not a promising monotherapy for glioblastoma. At the same time, both we and Fornvik *et al.* detected a biological effect (albeit a negative one) of ITPP, suggesting that it may have an impact that could be exploited if combined with appropriate co-therapy.

For example, recently published studies on the ability of intracranially administered semapimod to significantly enhance RT cures of orthotopic GL261 tumors (17) may shed light on the failure of ITPP therapy. Semapimod is thought to suppress the metabolism and migratory function of brain macrophage microglia, which have been shown to support the growth and RT resistance of glioblastoma cells. It is possible that ITPP is supportive of microglia following radiation, and that this has the effect of enhancing GL261 growth and making tumor cells more resistant to RT. This might also explain the faster growth of subcutaneously injected RG2 cells in the Fornvik *et al.* study. If ITPP continues to show dramatic beneficial impact on other solid tumors, especially in current clinical trials, it might be worth pursuing as a component of GBM therapy, despite the negative results reported here. Eventually it may be possible to obtain antitumor effects of ITTP in tumor cells while simultaneously countering its negative effects if these are due to a supportive impact on microglia with semapimod or another agent.

Conflicts of Interest

The Authors have no conflicts of interest related to this publication.

Acknowledgements

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