Overall survival in malignant glioma is significantly prolonged by neurosurgical delivery of etoposide and temozolomide from a thermo-responsive biodegradable paste.

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Running title

Long-term survival from intra-cavity biodegradable paste.

Keywords

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Additional information

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Abstract

Purpose

High-grade glioma (HGG) treatment is limited by the inability of otherwise potentially efficacious drugs to penetrate the blood brain barrier. We evaluate the unique intra-cavity delivery mode and translational potential of a blend of poly(*DL*-lactic acid-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) paste combining temozolomide and etoposide to treat surgically resected HGG.

Experimental Design

To prolong stability of temozolomide pro-drug, combined *in vitro* drug release was quantitatively assessed from low pH-based PLGA/PEG using advanced analytical methods. *In vitro* cytotoxicity was measured against a panel of HGG cell lines and patient-derived cultures using metabolic assays. *In vivo* safety and efficacy was evaluated using orthotopic 9L gliosarcoma allografts, previously utilized pre-clinically to develop Gliadel®.

Results

Combined etoposide and temozolomide *in vitro* release (22 and 7 days respectively) was achieved from a lactic acid-based PLGA/PEG paste, used to enhance stability of temozolomide prodrug. HGG cells from central-enhanced regions were more sensitive to each compound relative to primary lines derived from the HGG invasive margin. Both drugs retained cytotoxic capability upon release from PLGA/PEG. *In vivo* studies revealed a significant overall survival benefit in post-surgery 9L

orthotopic gliosarcomas, treated with intra-cavity delivered PLGA/PEG/temozolomide/etoposide and enhanced with adjuvant radiotherapy. Long-term survivorship was observed in over half the animals with histological confirmation of disease-free brain.

Conclusions

The significant survival benefit of intra-cavity chemotherapy demonstrates clinical applicability of PLGA/PEG paste-mediated delivery of temozolomide and etoposide adjuvant to radiotherapy. PLGA/PEG paste offers a future platform for combination delivery of molecular targeted compounds.

Translational Relevance

The clinical benefit of intra-cavity chemotherapy delivery after neurosurgical resection in glioblastoma has previously been demonstrated in phase III trials of Gliadel®. However, survival benefits are modest, in part due to the inability of pre-formed Gliadel® wafers to be applied at close proximity to the irregular infiltrative tumor margin and due to Gliadel® (and most pre-clinical drug delivery systems) only being able to deliver single agents to a highly heterogeneous disease. Furthermore, local release formulations of standard-of-care temozolomide have been hampered by compound instability and systemic delivery of etoposide has shown limited efficacy.

We have developed a self-assembling polymer microparticle paste which can be molded onto neurosurgical cavity lining, wherein temozolomide half-life is prolonged. Etoposide in combination with temozolomide (+/- adjuvant radiotherapy), shows a significant survival benefit *in vivo* with long-term disease-free survivors, relative to standard-of-care. A combined temozolomide/etoposide-releasing polymer paste may translate clinically as a second-generation intra-cavity drug delivery system for high-grade resectable glioma.

Introduction

WHO (World Health Organisation) IV high-grade gliomas (HGG) represent the most aggressive and genetically heterogeneous group of primary brain tumors. The most common sub-type is grade IV glioblastoma multiforme (GBM) with an age standardised global incidence of 4.6/100,000/year (1). The median survival for patients diagnosed with GBM remains dismal at 14.6 months and has not improved in recent years (2,3), despite advances in neuroimaging, surgery, radiotherapy and chemotherapy (4). The slight improvement in terms of patient survival for high-grade gliomas, does not match the general trend in cancer survival over the past two decades (5). Techniques such as the use of 5-aminolevulinic (5ALA)-based fluorescence-guided neurosurgery (Gliolan^{TM}) have improved rates of gross total resection and increased progression-free survival (6,7), but infiltrative disease remains within adjacent brain parenchyma and is responsible for tumour re-growth.

The efficacy of systemic chemotherapy is limited for many reasons, but one important limiting factor is the blood brain barrier (BBB), which typically restricts therapeutic concentrations from being delivered within the microenvironment of residual post-surgical neoplastic cells. Systemic toxicities are dose limiting with sub-therapeutic doses favouring acquisition of secondary resistance by GBM cells (8,9).

Temozolomide (TMZ) is the primary systemic chemotherapy agent used in the treatment of GBM at a dose of 150-200 mg/m^2, but penetration beyond the BBB

remains a limiting factor for efficacy (10,11). Higher doses of TMZ are proscribed due to dose-limiting bone marrow suppression with severe leukopenia and thrombocytopenia; however only minimal adverse neurological affects have been observed. Coupled to no requirement for hepatic drug activation, this collectively supports consideration of TMZ as an ideal candidate for direct local delivery to the brain (12).

Many potential strategies are being investigated to enhance drug penetration to the tumour microenvironment (13). Intra-cavity depot drug delivery systems represent one such strategy, which can be implanted at the time of maximal neurosurgical resection to deliver agents directly to the brain tissue. This method allows for potential delivery of high local drug doses to the residual infiltrative cells with minimised systemic exposure. This localised drug release approach is currently used in the Food and Drug Administration (FDA)- and National Institute for Health and Care Excellence (NICE)-approved treatment of GBM with Gliadel® wafers containing 3.85% carmustine (BCNU), demonstrated to have a modest, but significant positive effect on patient overall survival in randomised phase III trials (14,15). It is important to note that Gliadel® serves as the rationale that intra-cavity drug delivery, with a strategy reliant on diffusion and mass transport mechanisms away from the surgical cavity, is both viable and successful, representing one of only a very few therapies clinically-approved for GBM. Multiple phase III trials of targeted agents based on biological data have yet to show any overall survival benefit (16,17).

We have previously reported the clinical utility of a novel intra-cavity drug delivery self-assembling system, applied intra-operatively as a polymer micro-particulate paste

that molds to the neurosurgical resection cavity and sinters at body temperature (18). The glass transition temperature of the biodegradable polymer micro-particles made from poly(*DL*-lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) has been tuned to allow the particles to fuse, such that at 37°C the polymer paste solidifies (19). Application of a chemotherapeutic paste represents a novel mode of intra-cavity delivery, distinct from existing approaches, permitting close apposition to the irregular-shaped resection cavity lining and potentially minimizing effective drug diffusion distance into the invasive tumor margin and brain parenchyma beyond. Whilst thermo-sensitive solutions which form a gel upon interstitial delivery have been previously reported by us in pre-clinical studies, these do not offer comparable close contact to the surgical cavity lining and have yet to demonstrate combination drug release (20,21)

In vivo proof-of-concept was initially demonstrated by the incorporation of etoposide (ETOP) into PLGA/PEG paste and efficacious delivery to a subcutaneous GBM xenograft (22). As combination therapy clinical trials with systemically-delivered ETOP, a topoisomerase II inhibitor, have shown poor response rates attributed to poor BBB penetration and dose-limiting toxicities (23,24), intra-cavity delivery of ETOP warrants investigation.

As an increasing appreciation of the degree of intra-tumor molecular heterogeneity and sub-clonal divergence warrants considerations for multi-agent drug delivery, we now present a revised PLGA/PEG formulation incorporating TMZ and ETOP for simultaneous combination drug release. TMZ is delivered as a prodrug that is stable at low pH, but at higher pH spontaneously hydrolyses. The active hydrolysis product

MTIC [3-methyl-(triazen-1-yl)imidazole-4-carboxamide] rapidly breaks down to the reactive methyldiazonium ion (diazomethane) that alkylates DNA. (25). The spontaneous nature of TMZ metabolism allows some bioavailability in the tumor via oral administration as there is no requirement for first pass metabolism (26). However, plasma half-life of TMZ is low (1.24h) (27) and therefore intra-cavity administration has the potential to increase exposure of tumor cells to the active drug. Previous studies examining local release of TMZ have typically measured TMZ prodrug release within a neutral pH saline/water environment over a period of several days, thus failing to directly address TMZ instability (12,28). ETOP has been shown by our group and several others to be efficacious against high-grade glioma when delivered locally and/or in a targeted manner (22,29–32).

Here, we report a PLGA/PEG formulation tailored to incorporate active TMZ within a low pH environment and demonstrate precise *in vitro* quantitative release of TMZ in combination with ETOP over several weeks. Furthermore, we demonstrate tolerability and significantly prolonged overall survival, compared to standard-of-care treatment, in an aggressive, immunocompetent orthotopic glioma allograft model, previously utilized pre-clinically to develop Gliadel®. Our data highlights a potential therapeutic role in the treatment of high-grade glioma, for intra-cavity delivery of TMZ/ETOP combination therapy via PLGA/PEG paste. Furthermore, our platform technology is applicable for the consideration of rational combinations of next-generation targeted therapeutics.

Materials and Methods

PLGA/PEG microparticle matrix formulation

Thermosensitive particles were fabricated from blends of 53kDa P_{DL} LGA (85:15 DLG 4CA) (Evonik Industries) and PEG 400 (Sigma Aldrich, UK) as previously described (18). Briefly, a mixture of 93.5%:6.5% PLGA/PEG (w/v) was blended at 80-90°C on a hotplate, mixed and allowed to cool. Cooled polymer was then ground into particles and sieved to obtain the 100-200µm particle size fraction.

Matrix preparation for *in vitro* release

200mg of PLGA/PEG microparticles were mixed with 0.05% L-Lactic acid solution (Sigma-Aldrich, UK) containing 1.5mg of either TMZ or a combination of TMZ with ETOP (both Sigma-Aldrich, UK), at 1.5mg of each drug. The amount of solution was in the ratio of 1.0:0.8 (polymer:carrier solution), where 160µl of L-Lactic acid solution was used as a low pH (~3) carrier to increase half-life of the TMZ pro-drug and to form the microparticle paste at room temperature. L-Lactic acid as a carrier ensures greater stability of the TMZ pro-drug within PLGA/PEG; upon release from the polymer, TMZ converts by hydrolysis to MTIC (intermediate compound) and its active component, AIC, which is highly unstable at neutral pH. The paste was then applied into 3 cylindrical PTFE molds (4mm x 6mm) and incubated for 2 hours at 37°C in a humidified incubator. The resulting matrices contained 500µg for single TMZ and 1000µg for dual release studies (i.e. 500µg for each drug).

PLGA microsphere formulation

An emulsion of PLGA containing active ETOP was prepared and stabilized in an aqueous hardening bath. 50-100µm diameter. PLGA microspheres were harvested and mixed with PLGA/PEG thermosensitive microparticles and saline to form a microparticulate paste at room temperature, which sintered at 37°C to form a solidified matrix.

In vitro single and combination drug release

Triplicate scaffolds loaded with TMZ or TMZ and ETOP, were placed in 1mL of distilled water and incubated at 37°C. At given time intervals, water was removed, retained and replaced with 1ml fresh distilled water. The retained fraction was assayed using liquid chromatography-mass spectrometry (LC-MS) for combined TMZ/ETOP release (Applied Biosystems, California, USA). Non-drug loaded matrices containing 0.05% L-Lactic acid solution were used to test background absorbance.

Liquid chromatography-mass spectrometry (LC-MS) analysis of TMZ

Chromatographic TMZ separation was achieved using a Kinetex C18 50mm x 4.6mm 2.6 µm and a SecurityGuard cartridge C18 3 mm guard column (Phenomenex, California, USA) maintained at 30°C. Analytes were eluted with HPLC grade (Sigma-Aldrich, UK) mobile phases comprising 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, with a flow rate was 0.5mL/min. An API4000 triple quadrupole LC–MS/MS (Applied Biosystems) was used for analysis with electrospray ionization performed in positive ion mode with the following source

settings: curtain gas, 20; ion source gas 1, 50; ion source gas 2, 40; ion spray voltage, 5500; collision gas, 6; entrance potential, 10; ionization temperature, 500°C. Detection of TMZ was achieved using the transition m/z 195.088->138.0 in positive electrospray MRM mode. A standard curve over the range 0.44–500ng/ml TMZ in matched matrix was prepared fresh on each day of analysis.

LC-MS analysis of ETOP

Chromatographic separation of ETOP was achieved using a Perkin Elmer 200 Series HPLC with a Genesis C18 120Å 4 µ, 100mm (Kinesis Ltd, UK) and a SecurityGuard cartridge C18 3mm guard column (Phenomenex, California, USA) maintained at 30°C. Analytes were eluted with HPLC grade (Sigma-Aldrich, UK) mobile phases 50:50 (w/w) Acetonitrile:0.1% Ammonium Acetate pH (4.7) under isocratic flow of 0.3 ml/min. A 3200 QTrap LC–MS/MS (Applied Biosystems) was used for analysis with electrospray ionization performed in positive ion mode with the following source settings: curtain gas, 20; ion source gas 1, 70; ion source gas 2, 80; ion spray voltage, 5500; collision gas, 2; entrance potential, 10; ionization temperature, 500°C. Detection of ETOP was achieved using the transition m/z 674.332->229.1 in positive electrospray MRM mode. For sample analyses, a standard curve over the range 1.56–50 µg/ml ETOP was prepared in matched matrix.

***In vitro* cytotoxicity**

U-373MG (GBM) and 9L (rat gliosarcoma grade IV) cell lines were cultured in 1g/L glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich),

supplemented with 10% fetal bovine serum (FBS) (GE Healthcare) and 1% L-glutamine (Sigma-Aldrich). GIN27, GIN28 and GIN31 are Glioma INvasive margin GBM cell lines derived from the infiltrative margin of adult patients undergoing 5-aminolevulinic acid fluorescence-guided neurosurgical resection at the Queen's Medical Centre, University of Nottingham (Supplementary Table 1). GIN lines were derived from right temporal, right frontal and right temporal anatomical regions respectively and cultured in 1g/L glucose DMEM supplemented with 15% FBS and 1% L-glutamine. Cell line authentication of the U-373MG established line and GIN-27, GIN-28, GIN-31 patient-derived primary lines were determined by PCR-single-locus-technology, utilizing 21 independent PCR-systems (Eurofins, Germany) (Supplementary Figure 1). To assess acute cytotoxicity to TMZ and ETOP *in vitro*, all 6 cells lines were seeded onto 96-well plates at a density of 2-8 $x10^3$ cells/well, due to variability in cell size for each line. After 24 hours, cells were exposed to either serial dilutions of TMZ (concentration range 0 –2000μM) in triplicate wells, or ETOP (concentration range 0–200μM). Untreated and dimethyl sulfoxide (DMSO) (Sigma-Aldrich) carrier-only wells served as controls for metabolic viability. To mitigate against the short half-life of TMZ, fresh compound was added after 24 and 48 hours. Upon 72 hours acute exposure to either compound, PrestoBlue (Thermo Fisher) assay was conducted according to manufacturer instructions and fluorescence read using a FLUOstar Omega Microplate Reader (BMG Labtech.) (excitation 544nm; emission 590nm). Percentage metabolic viability was determined for each drug dose, relative to untreated cells and normalized for DMSO, with standard error of the mean calculated. To determine whether exposure of TMZ or ETOP to PLGA/PEG matrices impairs cytotoxic function, we designed an assay where glioma cells were directly exposed to drugs released from polymer. The 9L cell line was chosen for this analysis to compare

with 9L *in vivo* orthotopic data presented in this study. 1.25 x 10^4 9L cells/well were seeded onto a 12-well plate one day prior to PLGA/PEG-mediated drug release. PLGA/PEG matrices loaded with 500µg TMZ, ETOP or combined TMZ/ETOP (500µg per drug), were placed in NetWell inserts (Corning; 15mm) suspended over 9L cells, 24 hours post-seeding within 12-well plates. Cells were exposed to 48 hours drug release from PLGA/PEG and PrestoBlue assay used to determine metabolic viability. Combination indices (CI) to assess synergy for ETOP/TMZ exposure to 9L cells was determined using CompuSyn v1.0, based on the Chou-Talalay method (33).

Animals

Female F344 immunocompetent rats weighing 160-200 grams were purchased from Harlan Bioproducts and maintained in Individually Ventilated Cages (Harlan Bioproducts) within a barriered unit, illuminated by fluorescent lights set to give a 12-hour light-dark cycle (on 07.00, off 19.00), as recommended in the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (see Supplementary Methods for detailed animal welfare procedures). All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

***In vivo* safety and determination of PLGA/PEG/TMZ/ETOP maximum tolerated dose (MTD)**

A dose-escalation study was performed for locally delivered combinations of TMZ and ETOP to establish the MTD and toxicity. 9L gliosarcoma was maintained as a

subcutaneous mass and passaged every 3-4 weeks in the flanks of rats. After humane killing with an IP overdose of sodium pentobarbital (200mg/kg) (Butler Animal Health Supply), the tumor was surgically excised from the carrier animal and sliced into $2mm^3$ allografts. For intracranial implantation, rats were anesthetized with an intraperitoneal injection of 3mL/kg of a stock solution containing ketamine hydrochloride, 75mg/mL (Ketathesia, Butler Animal Health Supply), 7.5 mg/mL xylazine (Lloyd Laboratories) and 14.25% ethyl alcohol in 0.9% NaCl. All surgical procedures were performed using standard aseptic techniques, with sterile gloves, instruments and drapes used throughout the procedure. Animals were anaesthetized as above and the surgical area was shaved and prepped with ethanol and prepodyne. A midline scalp incision was made and a 3mm burr-hole was placed in the left parietal bone with its center 3mm lateral and 5mm posterior to bregma. A small incision was made through the dura and cortex and a small region of cortex resected. A $2mm^3$ allograft was placed in the resection cavity either 5 days prior to surgery and polymer implant (Batch 1) or concurrently (Batch 2). The wound was closed with sterile autoclips. For day 5 polymer implantation, the animal was anaesthetized as above. The previous incision was re-opened and a biopsy punch and fine suction tip used to resect the tumor back to the tumor-tissue interface, thereby mimicking the surgical technique utilized in human patients undergoing comparable GBM surgery. Animals were randomized into one of the following groups: Group 1 – Sham surgery (n = 2); Group 2 – Surgery/50mg PLGA/PEG (n = 6); Group 3 – Surgery/50mg PLGA/PEG containing 20% polymer weight %/drug weight % (w/w) TMZ (10mg) and 50% w/w ETOP (25mg) (n = 4); Group 4 – Surgery/50mg PLGA/PEG containing 10% w/w TMZ (5mg) and 25% w/w ETOP (12.5mg) (n = 4). Polymer microparticles and drugs were mixed at room temperature with PBS containing 0.05% L-Lactic acid to give a

mass:volume ratio of 1.0:0.8 for each formulation. Animals were evaluated post-operatively daily for 50 days and monitored for signs of toxicity, including weight loss, failure to thrive and neurological deficits (see Supplementary Methods for detailed animal welfare procedures).

In vivo efficacy of locally delivered PLGA/PEG/TMZ/ETOP

The MTD of 20% w/w TMZ and 50% w/w ETOP was chosen for all efficacy arms and 9L allografts were implanted 5 days prior to surgery and polymer implant. To ensure comparisons against clinical standard-of-care, per orem TMZ was given to animals at 50 mg/kg/day for 5 days (days 5-9) and radiotherapy (XRT) administered as an external beam single dose of 10Gy immediately after surgery. Rats were randomized into one of the following groups with n=7 per group: Group 1 – Untreated; Group 2 – Surgery/per orem TMZ/XRT (standard-of-care); Group 3 – Surgery/per orem TMZ; Group 4 – Surgery/XRT; Group 5 – Surgery/50mg PLGA/PEG; Group 6 – Surgery/50mg PLGA/PEG containing 20% w/w TMZ and 50% w/w ETOP; Group 7 – Surgery/50mg PLGA/PEG containing 20% w/w TMZ; Group 8 – Surgery/50 mg PLGA/PEG containing 50% w/w ETOP; Group 9 – Surgery/50mg PLGA/PEG containing 20% w/w TMZ and 50% w/w ETOP/XRT. Animals were evaluated post-operatively every day for up to 120 days and monitored for signs of adverse toxicity. Survival was assessed, animals euthanized and brains excised and stored in formalin after perfusion for histological analyses.

Histology and immunohistochemistry

Rat brains were fixed in 4% paraformaldehyde and 5µm sections obtained in a series proximal to the surgical resection boundary. Briefly, the slides were incubated at 37°C overnight, deparaffinized in xylene and hydrated through decreasing concentrations of ethanol. For Ki67 and CD31, antigen retrieval was performed in a pressure cooker for 7 minutes at full pressure in either sodium citrate buffer (pH 6.0) or TE buffer (pH 9.0). Sections were incubated with normal goat serum, followed by an endogenous peroxidase block (Dako, UK). Anti-Ki67 rabbit monoclonal antibody (Abcam, clone SP6, ab16667) and anti-CD31 rabbit polyclonal antibody (Abcam, ab28364) were incubated for 3 hours at room temperature (1:50). Target antigen was detected using the Dako Chemate Envision Detection Kit with diaminobenzidine chromogen for visualization, according to manufacturer instructions. Sections were counter stained with Harris hematoxylin (Surgipath, UK), dehydrated and mounted for microscopic analyses. For negative controls, primary antibody was replaced with antibody diluent.

Statistical analyses

In vitro cytotoxicity results are reported as the inhibitory concentration 50% (IC_{50}) values for each cell line given as the mean and standard error of the mean for three independent experiments, plotted relative to the % viability of vehicle-normalized untreated cells. Overall survival (OS) analyses were performed using SPSS v.14 (SPSS Inc.). OS was calculated from the time of surgery/polymer implant to the death from any cause. Kaplan-Meier survival curves with significance levels determined by the log-rank test were constructed by univariate analyses. p – values < 0.05 were deemed statistically significant. For *in vivo* efficacy, the Wilcoxon-Mann-Whitney

test was used to determine a sample size of n=7 per treatment arm, based on 80% power (5% significance; two-sided difference of means), where a standardized effect size (signal/noise ratio of 1.6) was estimated from tolerability studies comparing each individual treatment arm versus surgery only control.

Results

In vitro TMZ and combined ETOP/TMZ release from an acidic pH-based PLGA/PEG paste

The instability of TMZ (half-life 1.24h) presents difficulties when considering localized drug delivery at a single time-point. At neutral pH, the TMZ pro-drug spontaneously converts to the active hydrolysis product, MTIC. A low pH of ~3 is crucial in providing an environment in which TMZ is sufficiently stable to be incorporated within biomaterial formulations without rapidly degrading. PLGA/PEG microparticles mixed with 500µg TMZ and 0.05% organic (lactic) acid-based saline carrier (1.0:0.8 polymer:carrier), retained the ability to sinter at 37°C to form matrices (Figure 1A). *In vitro* release from matrices into saline, revealed that 70% of TMZ pro-drug was released on Day 1, 90% cumulatively after Day 2, continuing to complete drug release on Day 7, as determined by UV-Vis spectrophotometry and conservation of the TMZ molecular ion at 195.14 m/z further validated by LC-MS on Days 1 and 7 (Figure 1 B-D). This is consistent with TMZ release from biomaterial formulations previously reported by us (12,34). To confirm that PLGA/PEG matrices prepared with low pH carrier are capable of releasing drugs in combination with TMZ, matrices

were loaded with both TMZ and ETOP, released into saline and quantified using LC-MS. TMZ pro-drug burst release of 70% on Day 1 and complete release by Day 7 was observed, comparable to TMZ single release matrices. ETOP release showed a 60% Day 1 burst-release, followed by 80% cumulative drug release on Day 2 and a steady and gradual state release until total drug was released by Day 22 (Figure 1E), consistent with our previously reported ETOP release from PLGA/PEG with pH7 saline carrier (18). Whilst we have shown biomaterial flexibility of PLGA/PEG paste by encapsulating ETOP within PLGA microspheres which are mixed with the PLGA/PEG microparticles, thus overcoming burst release and permitting more controlled and sustained drug release (Supplementary Figure 2), we have taken forward the formulations with relatively higher burst release for *in vivo* therapy studies, to mitigate the aggressive nature of the 9L allograft.

Variable sensitivity of human and rodent high-grade glioma cells exposed to TMZ and ETOP *in vitro*

To assess the rationale for TMZ and ETOP localized delivery from PLGA/PEG paste, a panel of human GBM cell lines (representative of intra-tumor heterogeneity) was exposed to acute doses of either drug over 3 days. To validate the choice of the *in vivo* orthotopic model (9L grade IV gliosarcoma rat allografts) utilized in this study, the 9L cell line was exposed to a similar dosing regimen but which included TMZ and ETOP in combination.

U-373MG cells derived from the central-enhanced GBM core region, was acutely sensitive to ETOP with an IC_{50} concentrations of 0.4μM. Although a dose-dependent

decrease in metabolic viability was observed for all three GBM lines derived from the infiltrative margin, ETOP IC_{50} concentrations were not reached (Figure 2A). No IC_{50} concentrations were achieved for TMZ under any drug dose tested, although minimal but significant impaired metabolic viability was observed at 500µM TMZ after 72 hours for all cell lines (metabolic viability reduced by 10-20%) (Figure 2B). 9L gliosarcoma cells were sensitive to both drugs but markedly more sensitive to ETOP (IC_{50} <3.12µM relative to IC_{50} 500µM for TMZ). To determine combined effects of 9L exposure to ETOP and TMZ, the IC_{50} dose of TMZ (500µM) was added to individual wells containing an ETOP dose range of 0.39-100µM. Acute impairment of metabolic viability was observed over 72 hours in a similar manner to ETOP alone exposure, with an IC_{50} concentration of <3.12µM (39.4% viability +/- 0.9). (Figure 2C-E). According to the Chou-Talalay method to assess synergy, a combined dose of 500µM TMZ/6.25µM ETOP was 'nearly additive', with no evidence of synergy or additivity for any other dose combination (Supplementary Table 2). To confirm that incorporation of TMZ or ETOP into PLGA/PEG matrices does not impair cytotoxic capability, 9L cells exposed to TMZ, ETOP or combined TMZ/ETOP released from PLGA/PEG were assessed for metabolic viability relative to cells exposed to PLGA/PEG containing no drug. Drug doses (TMZ – 1mM; ETOP – 8.5µM) greater than the previously determined IC_{50} concentrations were chosen to ensure sufficient drug concentrations were achieved after 24 hours burst release. Released TMZ, ETOP and combined TMZ/ETOP resulted in 88.5% +/- 3.2, 50.0% +/- 1.0 and 45% +/- 2.2 metabolic viability respectively, confirming these agents retain cytotoxic capabilities when released from PLGA/PEG formulated with 0.05% lactic acid (Figure 2F).

In vivo tolerability of PLGA/PEG/TMZ/ETOP in orthotopic gliomas

To determine safety of intra-cavity delivery, PLGA/PEG/TMZ/ETOP paste was molded to the tumor cavity lining of rat brains immediately after surgical resection of 9L allograft gliomas (Figure 3A-B), which were either implanted 5 days prior to, or concurrently with polymer paste. Previous PLGA/PEG/TMZ/ETOP dosing to determine MTD in human GBM subcutaneous mouse xenografts, showed that 50mg PLGA/PEG containing either 15% w/w TMZ / 50% w/w ETOP or 20% w/w TMZ / 50% w/w ETOP, was well tolerated over 102 and 76 days respectively. A dose of 30% w/w TMZ / 50% w/w ETOP resulted in rapid loss of weight by day 60, indicating toxicity (data not shown). Based upon this MTD, two drug doses were assessed for orthotopic safety studies: (i) 50mg PLGA/PEG containing 10% w/w TMZ / 25% w/w ETOP; (ii) 50mg PLGA/PEG containing 20% w/w TMZ / 50% w/w ETOP. MTD was not reached as both doses were well tolerated over 2-3 weeks, with no difference in weight gain and other animal welfare measures observed relative to control animals treated with PLGA/PEG loaded with saline or animals undergoing surgery alone. Most of the control animals were sacrificed on Day 14 due to tumor-related adverse neurological deficits, whereas no neurological deficit was observed in PLGA/PEG/TMZ/ETOP animals (Figure 3C-D).

In vivo **efficacy of intra-cavity delivered PLGA/PEG/TMZ/ETOP in orthotopic glioma allografts**

At the termination of *in vivo* tolerability studies at Day 50 post surgery and polymer implant, both PLGA/PEG containing 10% w/w TMZ / 25% w/w ETOP or 20% w/w TMZ / 50% w/w ETOP, resulted in a significant survival benefit over sham surgery

and per orem TMZ controls ($P < 0.001$ and $P < 0.004$ when PLGA/PEG delivered 5-days post tumor implant or concurrently respectively). Only animals within PLGA/PEG treatment groups were still alive at Day 50 and treatment efficacy was confirmed histologically and via immunohistochemistry on post-sacrificial brain tissue (Supplementary Figures 3A-D and 4). As this was evident whether 9L allografts were implanted 5 days prior to surgery or on the day of surgery, the former tumor implantation time-point was selected for a therapy study as this more closely mimics the clinical scenario. Evidence of both extensive infiltration in the adjacent brain parenchyma close to the primary tumour site and individual infiltrative cells in the contra-lateral hemisphere, in animals receiving sham surgery, confirm the aggressive invasive nature of the 9L allograft (Supplementary Figure 3E-H).

To ensure statistical significance within a clinically-relevant powered therapy study, n=7 animals were used per treatment arm, adjuvant XRT was included and Day 120 post-treatment was regarded as a measure of long-term survivors (LTS) as previously reported by us (12,35). Intra-cavity delivery of PLGA/PEG/TMZ/ETOP consistently improved the survival of tumor-bearing animals compared to control arms. Untreated animals, animals that received surgery/blank polymer and animals that received surgery/per orem TMZ (Figure 4A and Table 1), had a median survival of 13.0, 12.0 and 19.0 days respectively. Animals that received surgery, per orem TMZ and XRT (Stupp protocol) had a relatively increased median survival of 26.0 days ($P < 0.0001$ vs. controls, surgery/blank polymer (Figure 4A) and surgery/XRT (Table 1). No significant difference was observed between the Stupp protocol and animals that received surgery/per orem TMZ (S/poT, n=7) ($P = 0.336$) (Figure 4A and Table 1). Animals that received either surgery/PLGA/PEG/TMZ/ETOP or

surgery/PLGA/PEG/ETOP, had an increased median survival of 33.0 and 71.0 days respectively compared to surgery/PLGA/PEG/TMZ ($P < 0.0001$ vs. controls; $P < 0.0001$ vs. surgery/PLGA/PEG/TMZ), with 28.6% LTS in each group (Figure 4B and Table 1). Animals that received surgery/PLGA/PEG/TMZ/ETOP with adjuvant XRT had an increased mean survival of 76.8 days relative to animals that received surgery/PLGA/PEG/TMZ/ETOP ($P < 0.0001$). Due to 4/7 LTS in the surgery/PLGA/PEG/TMZ/ETOP with adjuvant XRT group, median survival could not be determined (Figure 4C). A comparison of survival among all groups revealed animals receiving surgery/PLGA/PEG/TMZ/ETOP with adjuvant XRT had the greatest overall survival benefit, with 57.1% of animals deemed LTS, relative to 28.6% LTS for surgery/PLGA/PEG/TMZ/ETOP, 28.6% LTS for surgery/PLGA/PEG/ETOP and 14.3% LTS in animals treated with surgery and XRT (Table 1).

Histological and immunohistochemical confirmation of *in vivo* efficacy

To confirm that the observed survival benefit in PLGA/PEG treatment groups was directly due to efficacious intra-cavity delivery of TMZ/ETOP, histological and immunohistochemical analyses at the surgical margins and adjacent parenchyma were conducted on post-sacrificial brains. Untreated animals (Day 13), animals treated with surgery/per orem TMZ (Day 13), surgery/per orem TMZ/XRT (Day 22), surgery/XRT (Day 26) or surgery/blank PLGA-PEG (Day 14), all showed extensive tumor recurrence and dense cellularity within and surrounding the surgical resection cavity, with tumor cells visibly infiltrating brain parenchyma (Figure 5A-E). This finding is consistent with immunohistochemical staining for the proliferation marker

Ki67, where untreated animals (Day 13), animals treated with surgery/per orem TMZ (Day 13), surgery/per orem TMZ/XRT (Day 36), surgery/XRT (Day 26) or surgery/blank PLGA-PEG (Day 12), revealed high numbers of proliferative cells which have infiltrated into the surgical resection cavity and brain parenchyma (Figure 5I-M). In contrast, animals treated with surgery/PLGA-PEG-TMZ-ETOP (Day 120), surgery/PLGA-PEG-ETOP (Day 120) and surgery/PLGA-PEG-TMZ-ETOP/XRT (Day 120), show gliotic scarring but no histological/immunohistochemical evidence of recurrent proliferative tumor cells, consistent with long-term survivorship (Figure 5F-H; 5O-P). To further visualize treatment efficacy, histological staining on whole-brain cross-sections confirmed extensive 9L tumor regrowth and infiltration beyond the resection cavity in untreated and surgery/per orem TMZ animals. In marked contrast, LTS animals representative of surgery/PLGA/PEG/TMZ/ETOP and surgery/PLGA/PEG/TMZ/ETOP/XRT treatment groups, showed no evidence of tumor cells within and beyond the surgical resection cavity, confirming that these animals were likely disease-free (Supplementary Figure 5).

Discussion

Despite a substantial increase in clinical trials for high-grade glioma in the post-genomic era, particularly based on tumor subtyping revealed by integrated omics (36–38), no phase III efficacy has been reported for any molecular targeted therapy in a randomized trial. During this period, only an application of non-invasive, low intensity alternating electrical fields (Tumour Treating Fields; TTF) in combination with TMZ, has shown a significantly prolonged progression-free and overall survival

benefit, with FDA approval of TTF for the treatment of recurrent and newly diagnosed GBM (2011 and 2015 respectively) (39). The difficulty of delivering therapeutic doses across the BBB remains a substantial impediment to candidate molecular therapies. An increasing appreciation of intra-tumor heterogeneity and sub-clonal divergence, presents a compounding obstacle and highlights an urgent need to consider combination therapy in next-generation clinical trials (40–42).

Intra-cavity chemotherapy administered at the time of surgical resection using innovative and fit-for-purpose biomaterial formulations, continues to offer a means to bypass the BBB and deliver therapeutic concentrations in close proximity to infiltrative high-grade glioma. It also avoids the 'therapeutic void' between surgical resection and the start of adjuvant treatment, particularly radiotherapy, thereby preventing early tumor re-growth. It is important to note that the clinical success of Gliadel® not only provides a rationale for this delivery mode, but that such significant efficacy (albeit modest), has not been reported for any molecular targeted monotherapy trialed for GBM. Indeed, there has been a resurgence of efficacious intra-cavity delivery of repurposed agents using biomaterials in pre-clinical orthotopic brain tumor models (43–46).

In the current study, we have assessed *in vivo* efficacy of a novel intra-cavity delivery mode, whereby a biodegradable formulation of PLGA/PEG loaded with a combination of TMZ/ETOP is administered as a paste which molds to the irregular contours of a tumor resection cavity, sintering *in situ* and maintaining close conformity to the cavity lining. Our data reveals a significant overall survival benefit in surgery/PLGA/PEG/TMZ/ETOP treated 9L orthotopic gliosarcomas, with adjuvant

radiotherapy conferring long-term survivorship to more than half of the animals and histology confirming that LTS are disease-free. Our LTS is comparable to that of BCNU/carmustine previously reported as pre-clinical support for the clinical translation of Gliadel® and also comparable to LTS of BCNU/TMZ treatment arms reported recently, with both these studies utilizing the 9L orthotopic test-bed (47,48). Indeed survival in the 9L preclinical model with BCNU/carmustine was highly predictive of efficacy in a clinical trial with Gliadel®, validating this model as clinically-relevant for neurosurgically-applied drug delivery (49,50), with recent meta-analyses conducted on the safety and significant clinical efficacy (albeit modest), of Gliadel®(51,52). One must however be cautious in over-simplifying observed preclinical survival benefit with anticipated clinical benefit; whereas long-term survivorship was evident in the 9L/BCNU model, treatment with Gliadel® did not result in any long-term surviving patients during a phase III trial, with a modest median survival increase of 2.3 months relative to placebo-treated patients (15). Despite the 9L model likely over-predicting the clinical survival benefit of TMZ/ETOP delivered by PLGA/PEG in our study, the survival advantage to a subset of GBM patients when treated with Gliadel®, supports the consideration of our formulation for clinical trial and moreover presents a proof-of-concept for a Platform Technology with which to locally deliver other repurposed or experimental agents.

Although ETOP locally-delivered via convection enhanced delivery (CED) has been shown to be efficacious against malignant glioma (53) and is consistent with our finding, the previous report did not assess survival benefit against clinical standard of care (only versus untreated controls). Moreover, CED of ETOP was applied by intra-tumoral administration of catheters and not targeted to clinically-relevant post-

resection residual disease (53). Our findings warrant clinical translation consideration to test whether subsets of GBM patients may respond to localized ETOP therapy.

Whereas TMZ instability has rarely been considered for local delivery studies historically, we have prepared PLGA/PEG using an organic-acid based carrier to ensure TMZ is not rapidly converted to its active components until diffusion-mediated release from the polymer. Although *in vivo* release kinetics may differ, a low pH carrier does not impair *in vitro* burst and total release of combined TMZ/ETOP from PLGA/PEG. Despite surgery/PLGA/PEG/TMZ conferring a median survival advantage over surgery/per orem TMZ, including one LTS, 9L is more sensitive to ETOP. Surgery/PLGA/PEG/ETOP is comparably efficacious to surgery/PLGA/PEG/TMZ/ETOP, consistent with comparable IC_{50} concentrations from combined ETOP/TMZ or ETOP alone *in vitro*. As our *in vivo* study was restricted to one malignant glioma tumor model, this does not exclude the potential efficacy of low pH PLGA/PEG/TMZ against subsets of GBM with varying degrees of *MGMT* promoter methylation or other molecular resistance mechanisms. Indeed our formulation is applicable for next-generation TMZ analogue compounds (synthesized by our host institution), which have shown *in vitro* GBM cytotoxicity in a methylguanine-DNA methyltransferase-independent manner (54,55).

Although there is rationale for developing controlled and sustained release drug delivery formulations for GBM, it is likely that burst release of ETOP/TMZ (if *in vitro* release profiles are presumed to at least broadly be recapitulated *in vivo*) may have contributed to the observed efficacy. A high and local therapy dose with

sufficient tissue penetration, may therefore be effective against residual disease immediately post-surgery when tumor burden is relatively minimal.

Despite using ETOP MTD determined from our previous GBM subcutaneous xenograft study (22), ETOP MTD was not reached for orthotopic dosing, indicating that dose limiting systemic toxicities are avoided by intra-cavity delivery, with no evidence of neurotoxicity. This finding, coupled to the status of PLGA as an FDA-approved biodegradable medical implant material (56,57) with biocompatibility to brain tissue (58,59), indicates PLGA/PEG/ETOP/TMZ should be safe for clinical trial translation.

In summary, PLGA/PEG paste mediated intra-cavity delivery of ETOP/TMZ as an adjuvant to XRT has a substantial and significant overall survival benefit in an orthotopic *in vivo* glioma model with absence of detectable residual tumor associated with long-term survivorship. Our findings support repurposing ETOP for PLGA/PEG localized delivery and offers a future platform for combination delivery of molecularly targeted compounds. PLGA/PEG paste is in principle applicable to any solid cancer, for which surgical resection is standard-of-care and for which tumor recurrence is local in at least a subset of patients.

Author statement

RR, SJS and RGG conceptualized the study design; RR and SJS wrote the manuscript; BMT, GJV, AR, KMS, HB and RGG critically reviewed the manuscript; SJS, BMT, TG, GJV, NG, JR, RS, AR, PB, AO, JC, NS and RR generated

experimental data and supported the preparation of figures. All authors discussed the results and contributed to the final manuscript.

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Figure Legends

Figure 1: *In vitro* release of TMZ and combined TMZ/ETOP from an acidic pH formulation of PLGA/PEG paste. (A) Scanning electron images of PLGA/PEG microparticulate paste moulded into 12mm x 6mm diameter cylindrical matrices, each loaded with 500µg TMZ and 0.05% lactic acid. PLGA/PEG microparticles retain the ability to sinter at 37°C to form a matrix, despite the addition of an organic acid in the carrier phase used to disperse TMZ and create the paste. *Top – x50; Bottom – x500.* (B) *In vitro* cumulative release of TMZ pro-drug from PLGA/PEG matrices loaded with 500µg of drug. The release study was performed in PBS (pH 7.4) at 37°C and TMZ quantified using LC-MS for a 10-day period, at which time-point, all the drug was released. *Error bars indicate the standard error of the mean from three independent matrices*. (C-D) Mass spectrum of a sample measured at Day 1 and Day 7 of the *in vitro* release period showing the conservation of the TMZ molecular ion at 195.14 m/z (indicated by asterisks). (E) *In vitro* dual cumulative release of TMZ and ETOP from PLGA/PEG matrices loaded with 500µg of each drug and containing lactic acid carrier phase, was performed in PBS (pH 7.4) at 37°C and quantified using LC-MS. TMZ shows a 7-day release profile where total pro-drug has been released and ETOP shows a 22-day release profile. *Error bars indicate the standard error of the mean from three independent matrices*.

Figure 2: *In vitro* cytotoxicity of human and rodent high-grade glioma cells exposed to TMZ and ETOP. (A) A panel of human GBM cell lines were exposed to a concentration range of 0.01 – 500μM ETOP over 72 hours. Sensitivity to ETOP was determined by impairment of metabolic activity (PrestoBlue assay), relative to vehicle-controlled untreated cells, with an observed IC_{50} concentration of 0.4μM +/- 1.8 for U-373MG cells. Although a dose-dependent decrease in metabolic viability was observed for GIN-27, GIN-28 and GIN-31 lines derived from the GBM invasive margin, IC_{50} concentrations were not reached. (B) No IC_{50} concentrations were achieved for TMZ under any drug dose, although minimal but significant impaired metabolic viability was observed at 500μM TMZ after 72 hours: U-373MG – 79.9% viability +/- 5.5; GIN-27 – 89.0% viability +/- 3.5; GIN-28 – 86.5% viability +/- 3.02; GIN31 – 89.5% +/- 6.1. *A-B - Error bars represent standard error of the mean from the three independent experiments*. (C) Acute impairment of metabolic viability relative to vehicle-controlled untreated cells was observed in 9L rat gliosarcoma cells exposed to an ETOP dose range of 0.39-100μM over 72 hours, with an IC_{50} concentration of <3.12μM (36.% viability +/- 3.0). (D) Impaired 9L metabolic viability was observed within a TMZ dose range of 15.6-2000μM over 72 hours, with an IC_{50} concentration of 500μM (52.5% viability +/- 4.7). (E) To determine synergy, the IC_{50} dose of TMZ (500μM) was added to individual wells containing ETOP at 0.39-100μM for 72 hours, with an IC_{50} concentration of <3.12μM (39.4% viability +/- 0.9) observed. (F) 9L cells exposed to TMZ, ETOP or combined TMZ/ETOP released from PLGA/PEG matrices resulted in 88.5% +/- 3.2, 50.0% +/- 1.0 and 45% +/- 2.2 metabolic viability respectively, relative to cells exposed to PLGA/PEG containing no drug.

Figure 3: *In vivo* tolerability of PLGA/PEG/TMZ/ETOP in orthotopic gliomas. (A) Rat with surgically resected 9L tumor showing surgical cavity margins. (B) Rat with PLGA/PEG paste loaded with combined TMZ and ETOP, molded to the resection cavity lining. (C-D) Weight of rats measured daily for 15-20 days after surgery and polymer/drug implantation. *C = rats implanted with 9L allografts 5 days before therapy intervention; D = rats implanted with 9L allografts on day of therapy intervention. Control = surgery/PLGA/PEG; Sham = surgery alone; Low dose = Surgery/PLGA/PEG containing 10% w/w TMZ (5mg) and 25% w/w ETOP (12.5mg); High dose = Surgery/PLGA/PEG containing 20% w/w TMZ (10mg) and 50% w/w ETOP (25mg). n = number of animals per arm.*

Figure 4: *In vivo* efficacy of interstitially-delivered PLGA/PEG/TMZ/ETOP in orthotopic glioma allografts. Kaplan-Meier overall survival plots of F344 rats that were implanted with 9L and either given no treatment or were randomized and treated 5-days post allograft implant as follows: surgery + XRT + per orem TMZ by gavage (Stupp standard-of-care protocol, n=7); ; surgery + XRT; surgery + 50mg PLGA/PEG; surgery + 50mg PLGA/PEG containing 20% w/w TMZ and 50% w/w ETOP; Surgery + 50mg PLGA/PEG containing 20% w/w TMZ; Surgery + 50mg PLGA/PEG containing 50% w/w ETOP; surgery + 50mg PLGA/PEG containing 20% w/w TMZ and 50% w/w ETOP + XRT. (A) Animals that received surgery, per orem TMZ and XRT (S/R/poT) (Stupp protocol) had a relatively increased median survival compared to untreated animals (9L control, n=7) and animals that received surgery and blank polymer (S/blank, n=7) ($P < 0.0001$ for each comparison). No significant difference was observed between the Stupp protocol and animals that received surgery and per orem TMZ (S/poT, n=7) ($P = 0.336$). (B) Whilst animals that

received either surgery and PLGA/PEG/TMZ/ETOP (S/P/T&E, n=7), surgery and PLGA/PEG/ETOP (S/PE, n=7) or surgery and PLGA/PEG/TMZ (S/PT, n=7) had a relatively increased median survival compared to untreated animals (9L control, n=7) (P < 0.0001 for each comparison), animals that received either surgery and PLGA/PEG/TMZ/ETOP (S/P/T&E, n=7) or surgery and PLGA/PEG/ETOP (S/PE, n=7), had a relatively increased median survival compared to animals that received surgery and PLGA/PEG/TMZ (S/PT, n=7) (P < 0.0001 for each comparison). (C) Animals that received surgery and PLGA/PEG/TMZ/ETOP with adjuvant XRT (S/P/T&E/R, n=7) had an increased mean survival relative to animals that received surgery and PLGA/PEG/TMZ/ETOP (S/P/T&E, n=7) (P < 0.0001). *Animals alive at termination of experiment after 120 days post-surgery and polymer implant were deemed long-term survivors*.

Figure 5: Histological and immunohistochemical confirmation of efficacy after 120 days post-PLGA/PEG/TMZ/ETOP interstitial delivery to orthotopic 9L gliosarcomas. *Hematoxylin and eosin staining*: (A) Control untreated Day 13, (B) surgery/per orem TMZ Day 13, (C) surgery/XRT/per orem TMZ Day 22, (D) surgery/XRT Day 26 and (E) surgery/blank PLGA/PEG Day 14, show extensive tumor recurrence and dense cellularity (denoted by *) within the surgical resection cavity (delineated by arrowhead) with tumor cells visibly infiltrating brain parenchyma (denoted by +). (F) Surgery/PLGA/PEG/TMZ/ETOP Day 120, (G) surgery/PLGA/PEG/ETOP Day 120 and (H) surgery/PLGA/PEG/TMZ/ETOP/XRT Day 120, show gliotic scarring but no recurrent tumor cells. *Ki67 (proliferation marker) immunohistochemistry*: (I) Control untreated day 13, (J) surgery/per orem TMZ Day 13, (K) surgery/XRT/per orem TMZ Day 36, (L) surgery/XRT day 26 and

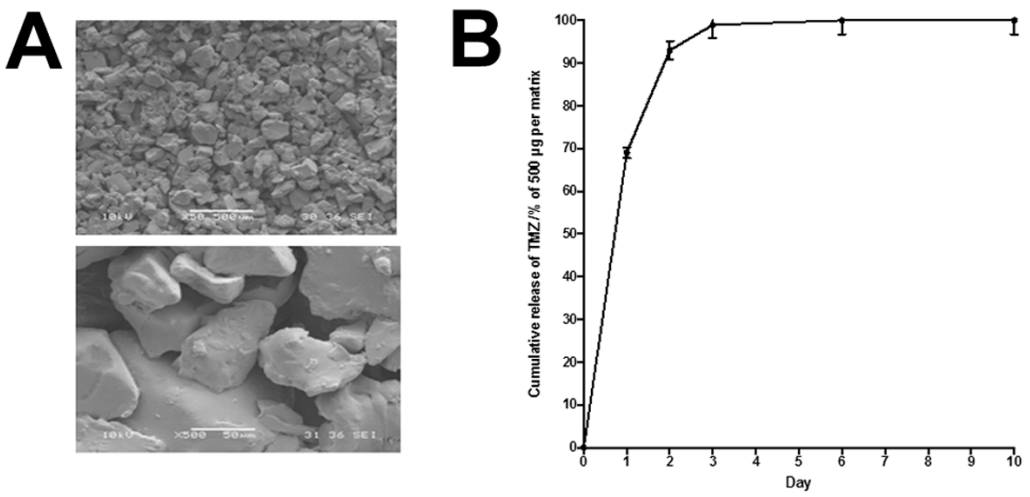
(M) surgery/blank PLGA/PEG Day 12, reveal high numbers of proliferative cells which have infiltrated into the surgical resection cavity. (N) Surgery/PLGA/PEG/TMZ/ETOP Day 120, (O) surgery/PLGA/PEG/ETOP Day 120 and (P) surgery/PLGA/PEG/TMZ/ETOP/XRT Day 120, show a surgical resection site with no visible proliferative cells. *All images taken at x40. Scale bar A-H 500µm. 'Days' = days post-polymer implant.*

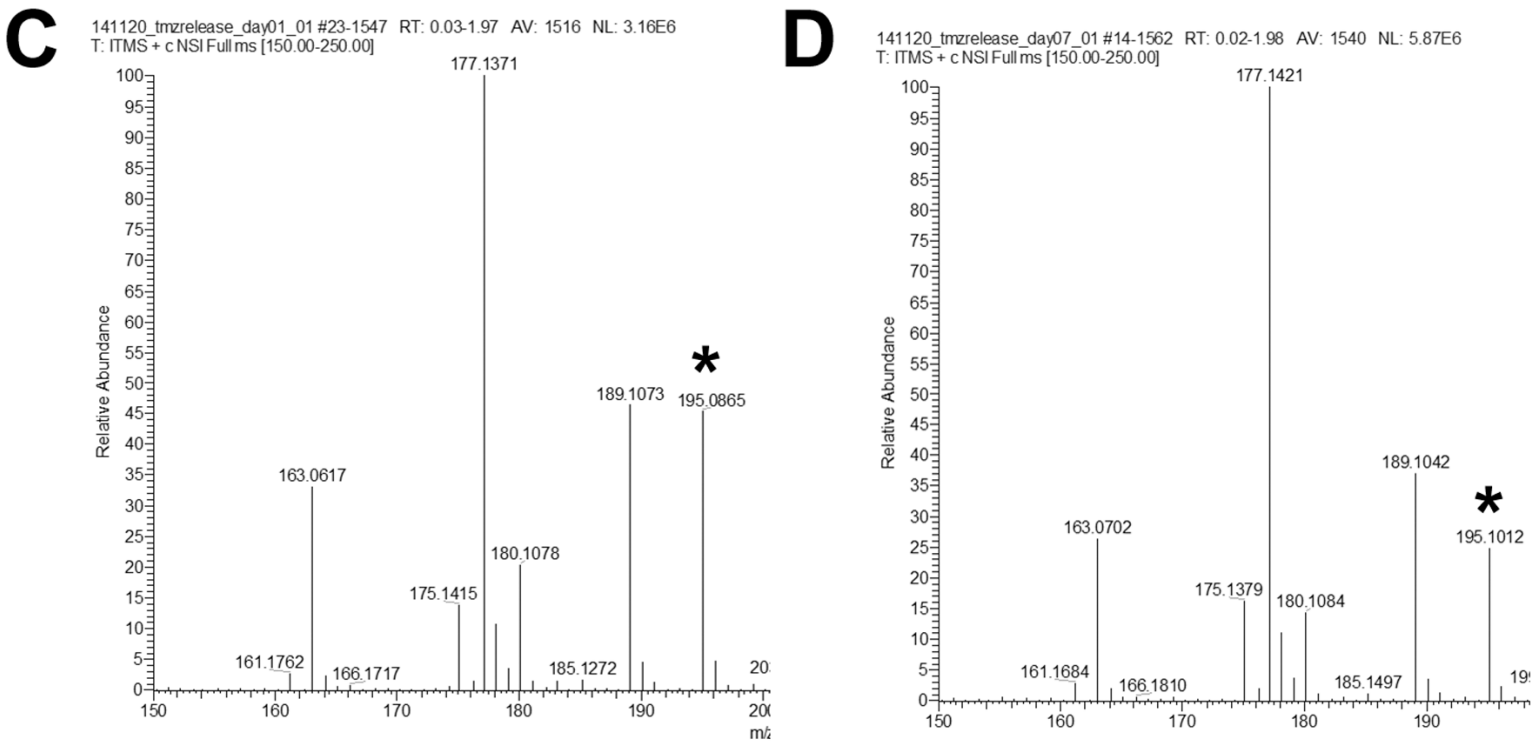
Table 1: Summary of median and mean overall survival in 9L orthotopic allografts treated with PLGA/PEG-delivered TMZ/ETOP +/- radiotherapy. Long-term survivors (LTS) are evident in PLGA/PEG treatment arms, relative to control arms with PLGA/PEG/TMZ/ETOP/XRT resulting in the highest relative percentage (57.1%) of LTS. [a] Estimation is limited to the largest survival time if it is censored. *Group labels - T, 10mg temozolomide; E, 25mg etoposide; S, surgery; R, irradiation (10Gy); poT, per orem temozolomide (50mg/kg/day for 5 days).*

Table 1

Group (n=7 per group)	Group (label)	Mean[a] Survival (days)	Median Survival (days)	Standard Error Mean	Long-term Survivors (LTR)	% LTR
All Groups Combined	All	42.8	31.0	11.9		
Untreated Control	Control	12.5	13.0	0.2	0	-
Surgery/XRT/Oral TMZ	S/R/poT	26.0	26.0	1.8	0	-
Surgery/Oral TMZ	S/poT	21.2	19.0	2.3	0	-
Surgery/XRT	S/R	35.7	23.0	13.0	1	14.3
Surgery/Blank PLGA-PEG	S/blank	12.8	12.0	0.8	0	-
Surgery/PLGA-PEG-TMZ-ETOP	S/PT&E	58.4	33.0	14.7	2	28.6
Surgery/PLGA-PEG-TMZ	S/PT	31.0	16.0	13.7	1	14.3
Surgery/PLGA-PEG-ETOP	S/PE	69.4	71.0	13.8	2	28.6
Surgery/PLGA-PEG-TMZ-ETOP/XRT	S/PT&E/R	76.8	N/A	18.9	4	57.1

Figure 1





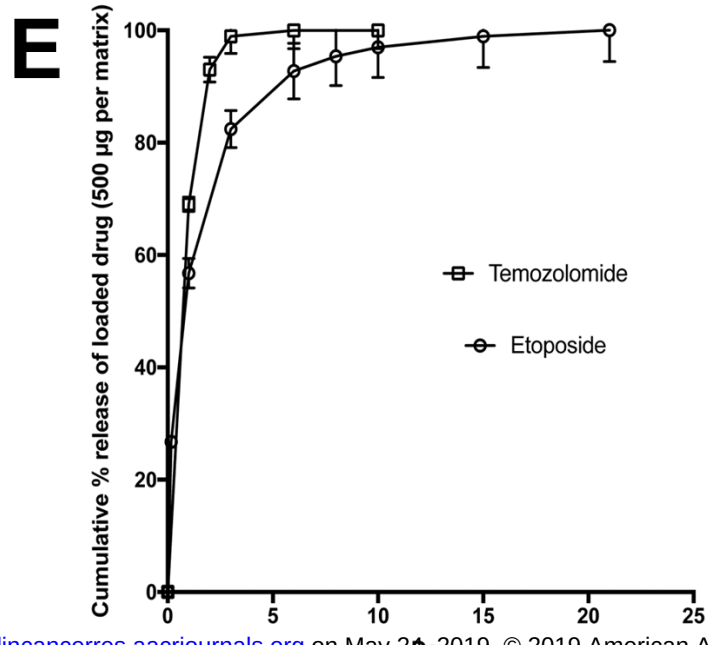
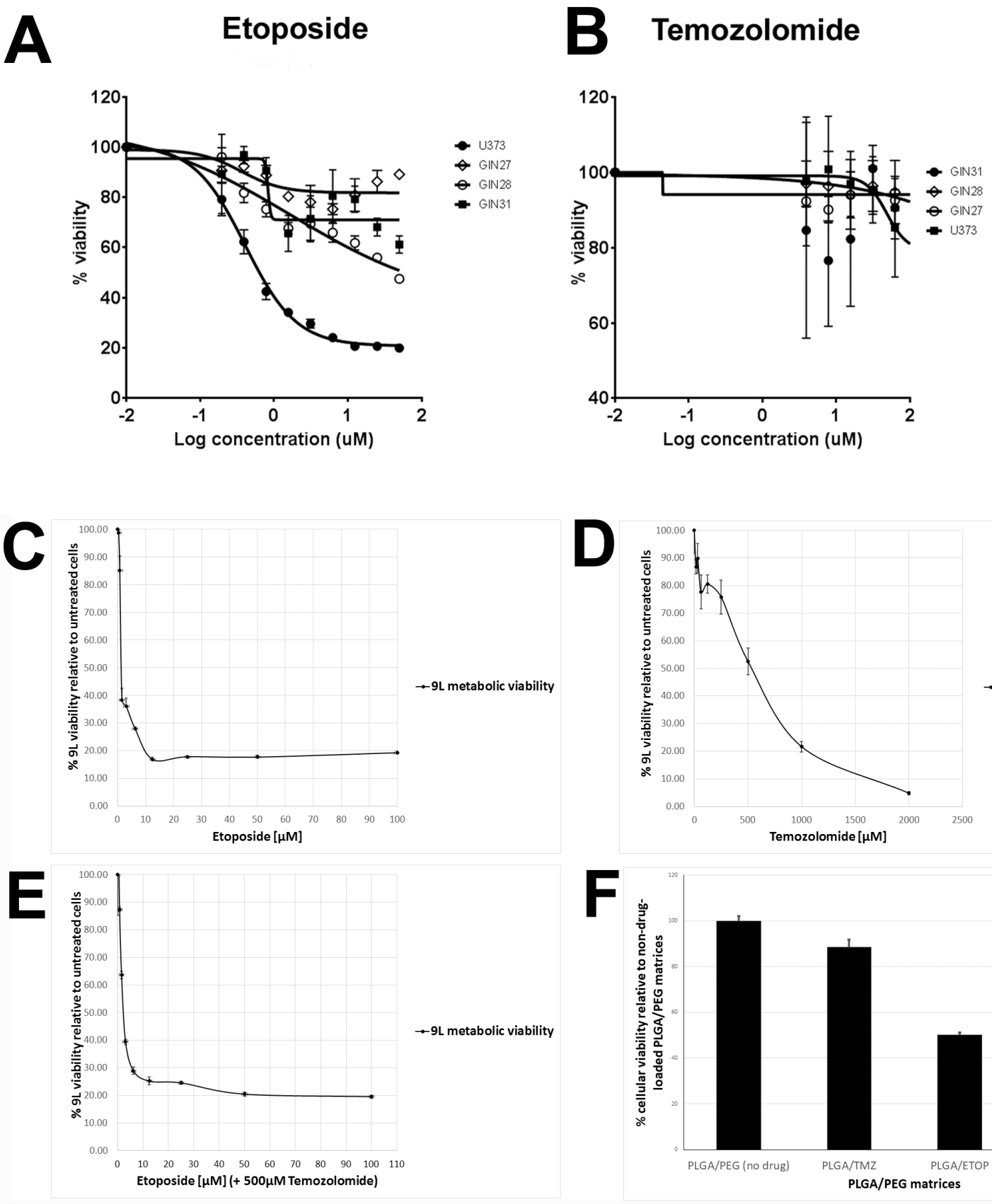


Figure 2



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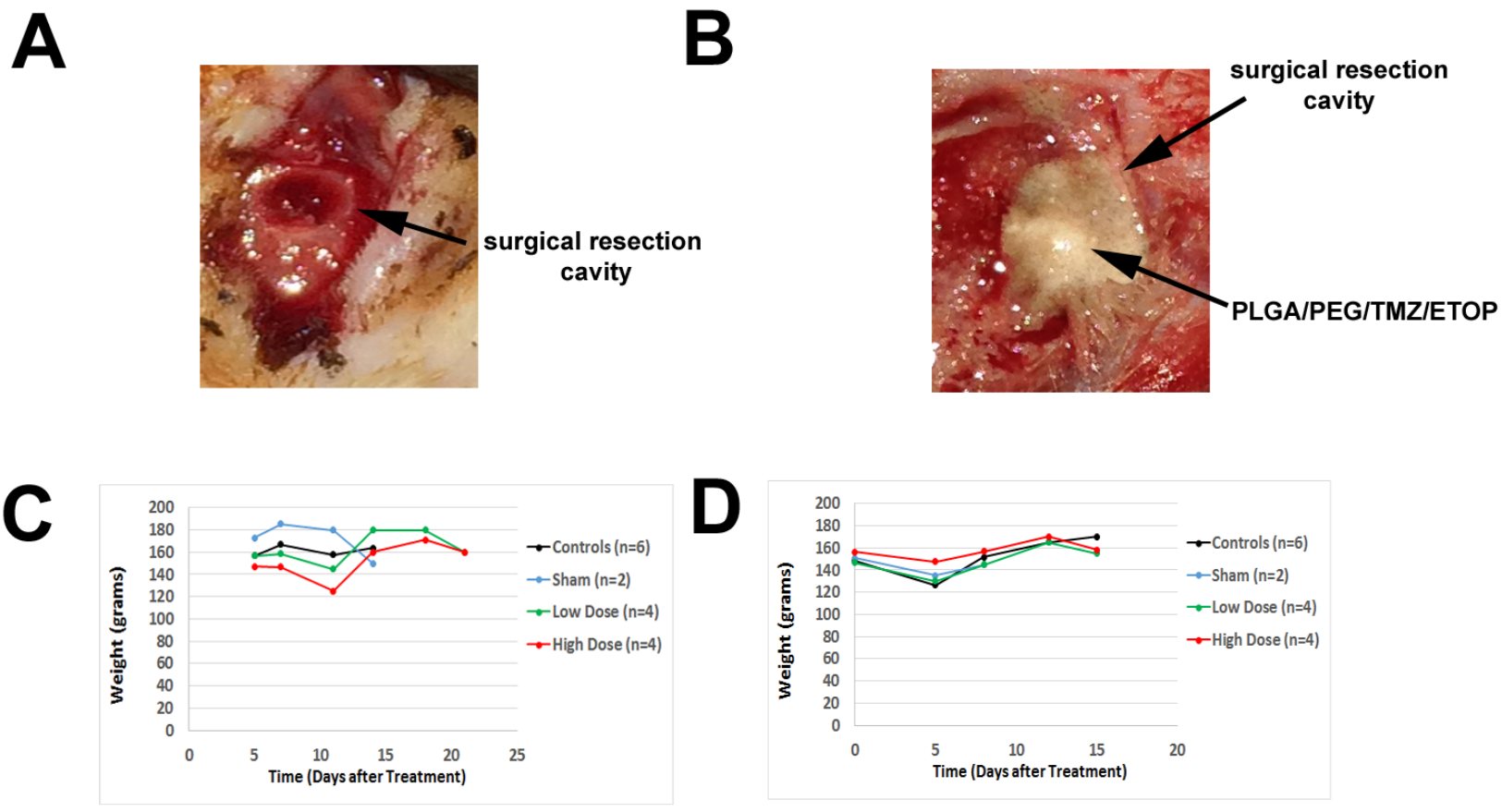
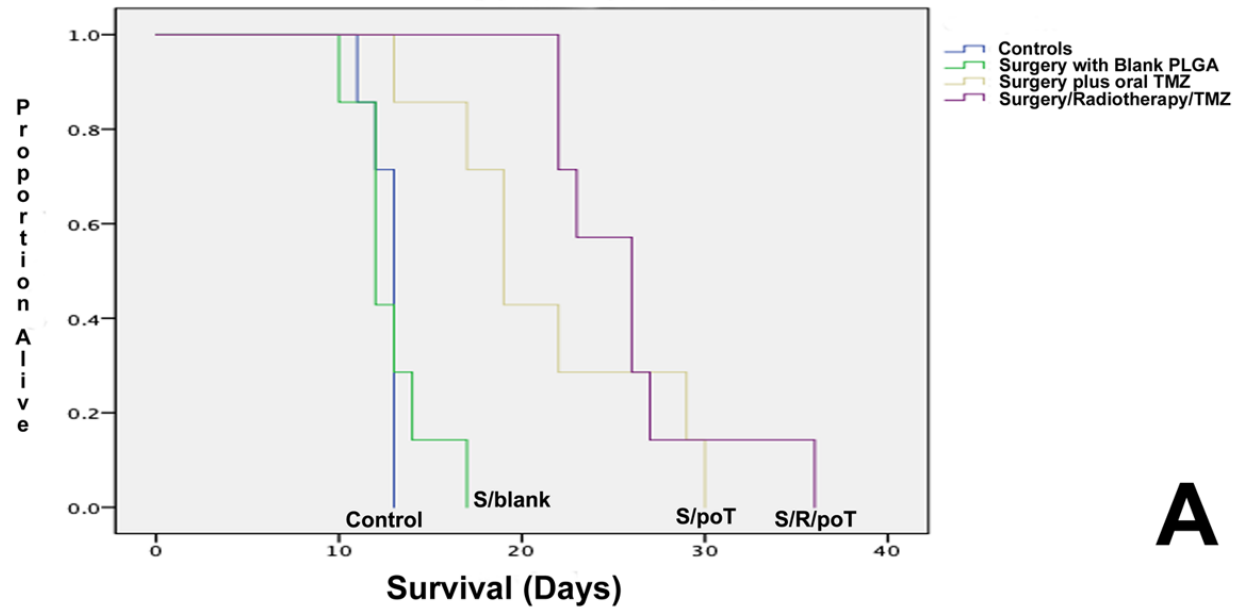


Figure 4



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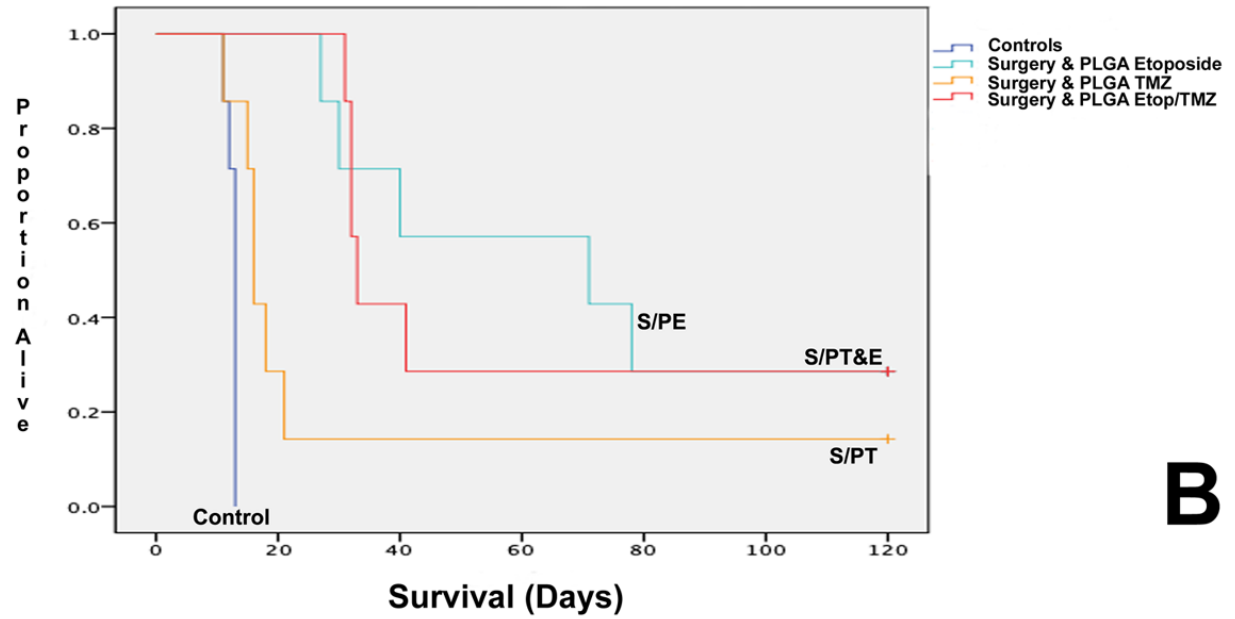

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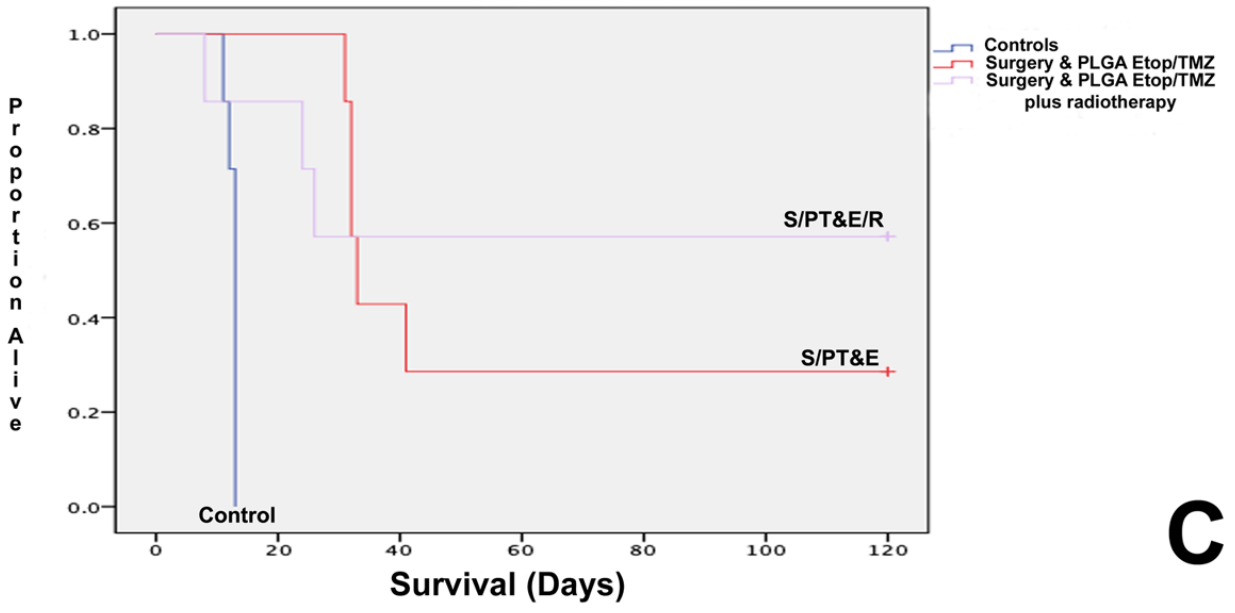
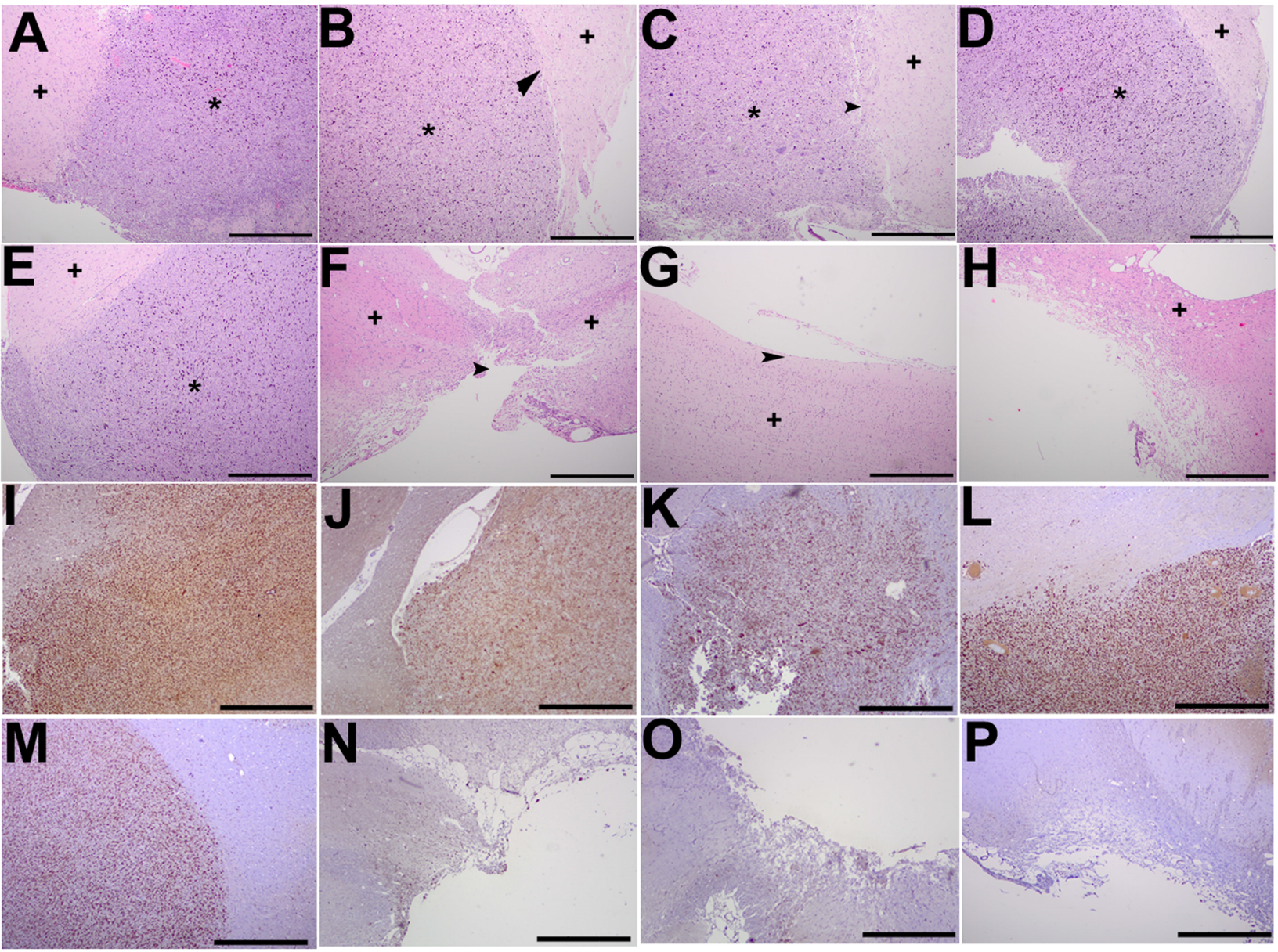


C

Figure 5



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