

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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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 intracavity 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 prodrug, combined *in vitro* drug release was quantitatively assessed from low pH–based PLGA/PEG using advanced analytic 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 preclinically 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 postsurgery 9L orthotopic gliosarcomas, treated with intracavity delivered PLGA/PEG/temozolomide/etoposide and enhanced with adjuvant radiotherapy. Long-term survivorship was observed in over half the animals with histologic confirmation of disease-free brain.

Conclusions: The significant survival benefit of intracavity 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.

Introduction

WHO (World Health Organization) IV high-grade gliomas (HGG) represent the most aggressive and genetically heterogeneous group of primary brain tumors. The most common subtype is grade IV glioblastoma multiforme (GBM) with an age-

standardized 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 2 decades (5). Techniques such as the use of 5-aminolevulinic (5ALA)-based fluorescence-guided neurosurgery (Gliolan) 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 tumor regrowth.

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 postsurgical neoplastic cells. Systemic toxicities are dose limiting with subtherapeutic doses favoring acquisition of secondary resistance by GBM cells (8, 9).

Temozolomide is the primary systemic chemotherapy agent used in the treatment of GBM at a dose of 150–200 mg/m², but penetration beyond the BBB remains a limiting factor for efficacy (10, 11). Higher doses of temozolomide are proscribed due to

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Translational Relevance

The clinical benefit of intracavity 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 preformed Gliadel wafers to be applied at close proximity to the irregular infiltrative tumor margin and due to Gliadel (and most preclinical 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 that can be molded onto neurosurgical cavity lining, wherein temozolomide half-life is prolonged. Etoposide in combination with temozolomide (\pm 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 intracavity drug delivery system for high-grade resectable glioma.

dose-limiting bone marrow suppression with severe leukopenia and thrombocytopenia; however, only minimal adverse neurologic effects have been observed. Coupled to no requirement for hepatic drug activation, this collectively supports consideration of temozolomide as an ideal candidate for direct local delivery to the brain (12).

Many potential strategies are being investigated to enhance drug penetration to the tumor microenvironment (13). Intracavity 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 minimized systemic exposure. This localized drug release approach is currently used in the 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 randomized phase III trials (14, 15). It is important to note that Gliadel serves as the rationale that intracavity 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 intracavity drug delivery self-assembling system, applied intraoperatively as a polymer microparticulate paste that molds to the neurosurgical resection cavity and sinters at body temperature (18). The glass transition temperature of the biodegradable polymer microparticles 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 intracavity 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. While thermosensitive solutions, which form a gel upon interstitial delivery, have been previously reported by us in preclinical 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 into PLGA/PEG paste and efficacious delivery to a subcutaneous GBM xenograft (22). As combination therapy clinical trials with systemically delivered etoposide, a topoisomerase II inhibitor, have shown poor response rates attributed to poor BBB penetration and dose-limiting toxicities (23, 24), intracavity delivery of etoposide warrants investigation.

As an increasing appreciation of the degree of intratumor molecular heterogeneity and subclonal divergence warrants considerations for multiagent drug delivery, we now present a revised PLGA/PEG formulation incorporating temozolomide and etoposide for simultaneous combination drug release. Temozolomide is delivered as a prodrug that is stable at low pH, but at higher pH spontaneously hydrolyses. The active hydrolysis product MTIC [3-methyl-(1H-tetrazol-5-yl)imidazole-4-carboxamide] rapidly breaks down to the reactive methyl diazonium ion (diazomethane) that alkylates DNA. (25). The spontaneous nature of temozolomide 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 temozolomide is low (1.24 hours; ref. 27) and therefore intracavity administration has the potential to increase exposure of tumor cells to the active drug. Previous studies examining local release of temozolomide have typically measured temozolomide prodrug release within a neutral pH saline/water environment over a period of several days, thus failing to directly address temozolomide instability (12, 28). Etoposide 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 temozolomide within a low pH environment and demonstrate precise *in vitro* quantitative release of temozolomide in combination with etoposide over several weeks. Furthermore, we demonstrate tolerability and significantly prolonged overall survival, compared with standard-of-care treatment, in an aggressive, immunocompetent orthotopic glioma allograft model, previously utilized preclinically to develop Gliadel. Our data highlight a potential therapeutic role in the treatment of high-grade glioma, for intracavity delivery of temozolomide/etoposide 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 53 kDa P_{DL} LGA (85:15 DLG 4CA; Evonik Industries) and PEG 400 (Sigma Aldrich) as described previously (18). Briefly, a mixture of 93.5%:6.5% PLGA/PEG (w/v) was blended at

80°C–90°C on a hotplate, mixed, and allowed to cool. Cooled polymer was then ground into particles and sieved to obtain the 100- to 200- μ m particle size fraction.

Matrix preparation for *in vitro* release

Two-hundred milligrams of PLGA/PEG microparticles was mixed with 0.05% L-Lactic acid solution (Sigma Aldrich) containing 1.5 mg of either temozolomide or a combination of temozolomide with etoposide (both Sigma-Aldrich), at 1.5 mg of each drug. The amount of solution was in the ratio of 1.0:0.8 (polymer:carrier solution), where 160 μ L of L-Lactic acid solution was used as a low pH (\sim 3) carrier to increase half-life of the temozolomide prodrug and to form the micro-particle paste at room temperature. L-Lactic acid as a carrier ensures greater stability of the temozolomide prodrug within PLGA/PEG; upon release from the polymer, temozolomide 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 three cylindrical PTFE molds (4 mm \times 6 mm) and incubated for 2 hours at 37°C in a humidified incubator. The resulting matrices contained 500 μ g for single temozolomide and 1,000 μ g for dual-release studies (i.e. 500 μ g for each drug).

PLGA microsphere formulation

An emulsion of PLGA containing active etoposide was prepared and stabilized in an aqueous hardening bath. Fifty- to 100- μ m-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 temozolomide or temozolomide and etoposide, were placed in 1 mL of distilled water and incubated at 37°C. At given time intervals, water was removed, retained, and replaced with 1-mL fresh distilled water. The retained fraction was assayed using LC/MS for combined temozolomide/etoposide release (Applied Biosystems). Nondrug-loaded matrices containing 0.05% L-Lactic acid solution were used to test background absorbance.

LC/MS analysis of temozolomide

Chromatographic temozolomide separation was achieved using a Kinetex C18 50-mm \times 4.6-mm 2.6- μ m and a Security-Guard cartridge C18 3-mm guard column (Phenomenex) maintained at 30°C. Analytes were eluted with high-performance liquid chromatography (HPLC)-grade (Sigma-Aldrich) mobile phases comprising 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, with a flow rate of 0.5 mL/minute. 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, 5,500; collision gas, 6; entrance potential, 10; ionization temperature, 500°C. Detection of temozolomide was achieved using the transition m/z 195.088 \rightarrow 138.0 in positive electrospray MRM mode. A standard curve over the range 0.44–500 ng/mL temozolomide in matched matrix was prepared fresh on each day of analysis.

LC/MS analysis of etoposide

Chromatographic separation of etoposide was achieved using a Perkin Elmer 200 Series HPLC with a Genesis C18 120Å 4- μ , 100-mm (Kinesis Ltd) and a SecurityGuard cartridge C18 3-mm guard column (Phenomenex) maintained at 30°C. Analytes were eluted with HPLC-grade (Sigma-Aldrich) mobile phases 50:50 (w/w) acetonitrile:0.1% ammonium acetate pH (4.7) under isocratic flow of 0.3 mL/minute. 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, 5,500; collision gas, 2; entrance potential, 10; ionization temperature, 500°C. Detection of etoposide was achieved using the transition m/z 674.332 \rightarrow 229.1 in positive electrospray MRM mode. For sample analyses, a standard curve over the range 1.56–50 μ g/mL etoposide was prepared in matched matrix.

In vitro cytotoxicity

U-373MG (GBM) and 9L (rat gliosarcoma grade IV) cell lines were cultured in 1 g/L glucose DMEM (Sigma-Aldrich), supplemented with 10% 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 (Nottingham, United Kingdom; Supplementary Table S1). GIN lines were derived from right temporal, right frontal, and right temporal anatomic regions, respectively, and cultured in 1 g/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; Supplementary Fig. S1). To assess acute cytotoxicity to temozolomide and etoposide *in vitro*, all 6 cell lines were seeded onto 96-well plates at a density of $2\text{--}8 \times 10^3$ cells/well, due to variability in cell size for each line. After 24 hours, cells were exposed to either serial dilutions of temozolomide (concentration range, 0–2,000 μ mol/L) in triplicate wells, or etoposide (concentration range, 0–200 μ mol/L). Untreated and DMSO (Sigma-Aldrich) carrier-only wells served as controls for metabolic viability. To mitigate against the short half-life of temozolomide, fresh compound was added after 24 and 48 hours. Upon 72-hour acute exposure to either compound, PrestoBlue (Thermo Fisher Scientific) assay was conducted according to the manufacturer's instructions and fluorescence read using a FLUOstar Omega Microplate Reader (BMG Labtech; excitation 544 nm; emission 590 nm). Percentage metabolic viability was determined for each drug dose, relative to untreated cells and normalized for DMSO, with SEM calculated. To determine whether exposure of temozolomide or etoposide 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. A total of 1.25×10^4 9L cells/well were seeded onto a 12-well plate one day prior to PLGA/PEG-mediated drug release. PLGA/PEG matrices loaded with 500 μ g temozolomide, etoposide, or combined temozolomide/etoposide (500 μ g per drug), were placed in NetWell inserts (Corning; 15 mm) suspended over 9L cells, 24 hours postseeding within 12-well plates. Cells were exposed to 48-hour drug release

from PLGA/PEG and PrestoBlue assay used to determine metabolic viability. Combination indices (CIs) to assess synergy for etoposide/temozolomide 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 g 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/temozolomide/etoposide MTD

A dose-escalation study was performed for locally delivered combinations of temozolomide and etoposide 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 intraperitoneal overdose of sodium pentobarbital (200 mg/kg; Butler Animal Health Supply), the tumor was surgically excised from the carrier animal and sliced into 2 mm³ allografts. For intracranial implantation, rats were anesthetized with an intraperitoneal injection of 3 mL/kg of a stock solution containing ketamine hydrochloride, 75 mg/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 anesthetized as above and the surgical area was shaved and prepped with ethanol and prepodyne. A midline scalp incision was made and a 3-mm burr hole was placed in the left parietal bone with its center 3 mm lateral and 5 mm posterior to bregma. A small incision was made through the dura and cortex and a small region of cortex resected. A 2-mm³ 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 anesthetized as above. The previous incision was reopened 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/50 mg PLGA/PEG ($n = 6$); group 3, surgery/50-mg PLGA/PEG containing 20% polymer weight %/drug weight % (w/w) temozolomide (10 mg) and 50% w/w etoposide (25 mg; $n = 4$); group 4, surgery/50-mg PLGA/PEG containing 10% w/w temozolomide (5 mg) and 25% w/w etoposide (12.5 mg; $n = 4$). 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 postoperatively daily for 50 days and monitored for signs of toxicity, including weight loss, failure to thrive, and neurologic deficits (see Supplementary Methods for detailed animal welfare procedures).

In vivo efficacy of locally delivered PLGA/PEG/temozolomide/etoposide

The MTD of 20% w/w temozolomide and 50% w/w etoposide 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* temozolomide was given to animals at 50 mg/kg/day for 5 days (days 5–9) and radiotherapy administered as an external beam single dose of 10 Gy 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* temozolomide/radiotherapy (standard-of-care); group 3, surgery/*per orem* temozolomide; group 4, surgery/radiotherapy; group 5, surgery/50-mg PLGA/PEG; group 6, surgery/50-mg PLGA/PEG containing 20% w/w temozolomide and 50% w/w etoposide; group 7, surgery/50-mg PLGA/PEG containing 20% w/w temozolomide; group 8, surgery/50-mg PLGA/PEG containing 50% w/w etoposide; group 9, surgery/50-mg PLGA/PEG containing 20% w/w temozolomide and 50% w/w etoposide/radiotherapy. Animals were evaluated postoperatively 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 histologic analyses.

Histology and IHC

Rat brains were fixed in 4% paraformaldehyde and 5- μ m 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). Anti-Ki67 rabbit mAb (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 the manufacturer's instructions. Sections were counter stained with Harris hematoxylin (Surgipath), 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% (IC₅₀) values for each cell line given as the mean and SEM for three independent experiments, plotted relative to the percent 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-to-noise ratio of 1.6) was estimated from tolerability studies comparing each individual treatment arm versus surgery-only control.

Results

In vitro temozolomide and combined etoposide/temozolomide release from an acidic pH-based PLGA/PEG paste

The instability of temozolomide (half-life 1.24 hours) presents difficulties when considering localized drug delivery at a single timepoint. At neutral pH, the temozolomide prodrug spontaneously converts to the active hydrolysis product, MTIC. A low pH of approximately 3 is crucial in providing an environment in which temozolomide is sufficiently stable to be incorporated within biomaterial formulations without rapidly degrading. PLGA/PEG microparticles mixed with 500- μ g temozolomide 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 (Fig. 1A). *In vitro* release from matrices into saline revealed that 70% of temozolomide prodrug 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 temozolomide molecular ion at 195.14 m/z further validated by LC/MS on days 1 and 7 (Fig. 1 B–D). This is consistent with temozolomide 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 temozolomide, matrices were loaded with both temozolomide and etoposide, released into saline and quantified using LC/MS. Temozolomide prodrug burst release of 70% on day 1 and complete release by day 7 was observed, comparable with temozolomide single-release matrices. Etoposide 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 (Fig. 1E), consistent with our previously reported etoposide release from PLGA/PEG with pH7 saline carrier (18). While we have shown biomaterial flexibility of PLGA/PEG paste by encapsulating etoposide within PLGA microspheres, which are mixed with the PLGA/PEG microparticles, thus overcoming burst release and permitting more controlled and sustained drug release (Supplementary Fig. S2), 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 temozolomide and etoposide *in vitro*

To assess the rationale for temozolomide and etoposide localized delivery from PLGA/PEG paste, a panel of human GBM cell lines (representative of intratumor 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 temozolomide and etoposide in combination.

U-373MG cells derived from the central-enhanced GBM core region, was acutely sensitive to etoposide with an IC₅₀ concentrations of 0.4 μ mol/L. Although a dose-dependent decrease in metabolic viability was observed for all three GBM lines derived from the infiltrative margin, etoposide IC₅₀ concentrations were not reached (Fig. 2A). No IC₅₀ concentrations were achieved for temozolomide under any drug dose tested, although minimal but significant impaired metabolic viability was observed at 500 μ mol/L temozolomide after 72 hours for all cell lines (metabolic viability reduced by 10%–20%; Fig. 2B). 9L gliosarcoma

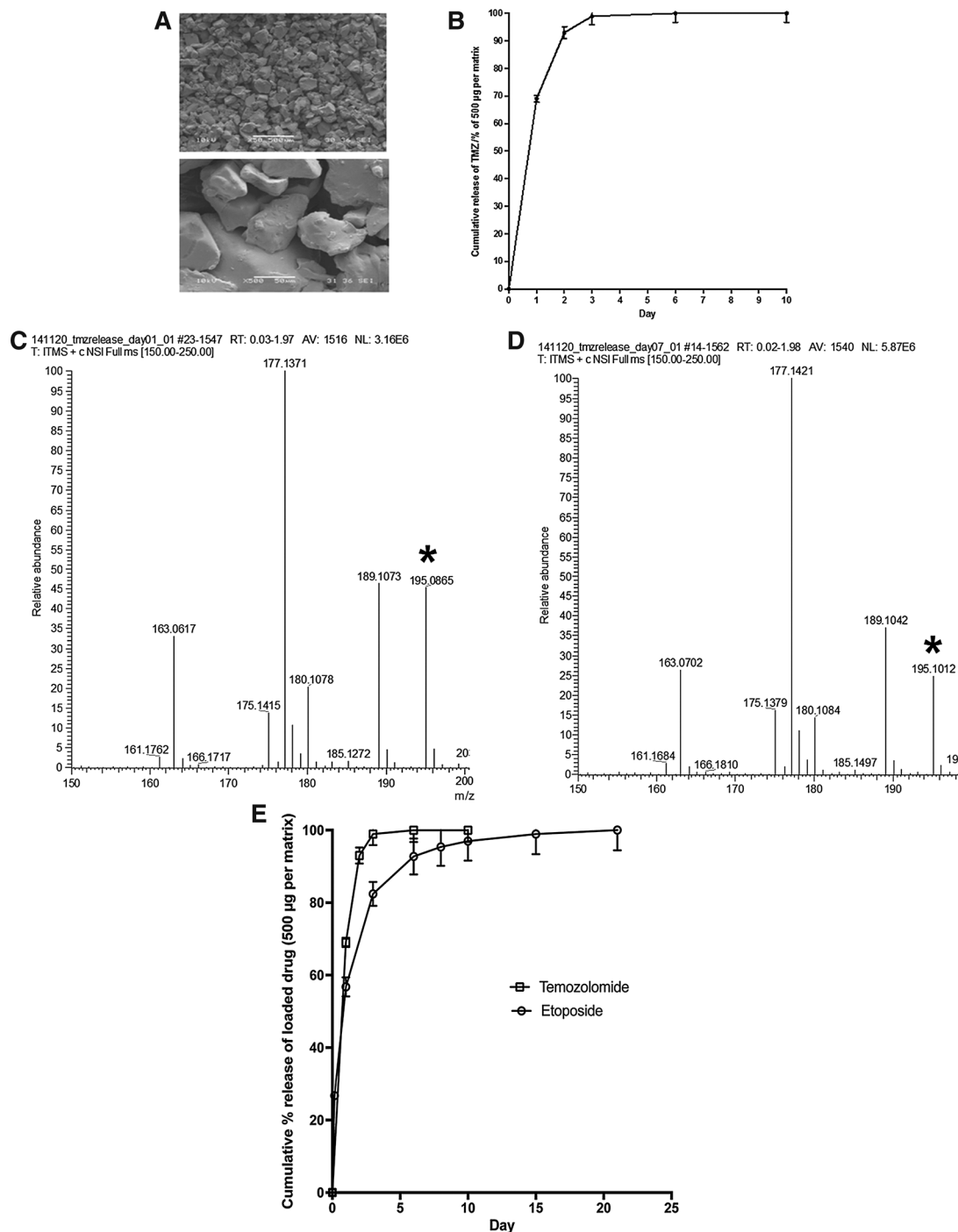
cells were sensitive to both drugs but markedly more sensitive to etoposide (IC₅₀ <3.12 μ mol/L relative to IC₅₀ 500 μ mol/L for temozolomide). To determine combined effects of 9L exposure to etoposide and temozolomide, the IC₅₀ dose of temozolomide (500 μ mol/L) was added to individual wells containing an etoposide dose range of 0.39–100 μ mol/L. Acute impairment of metabolic viability was observed over 72 hours in a similar manner to etoposide alone exposure, with an IC₅₀ concentration of <3.12 μ mol/L (39.4% viability \pm 0.9; Fig. 2C–E). According to the Chou–Talalay method to assess synergy, a combined dose of 500 μ mol/L temozolomide/6.25 μ mol/L etoposide was "nearly additive," with no evidence of synergy or additivity for any other dose combination (Supplementary Table S2). To confirm that incorporation of temozolomide or etoposide into PLGA/PEG matrices does not impair cytotoxic capability, 9L cells exposed to temozolomide, etoposide, or combined temozolomide/etoposide released from PLGA/PEG were assessed for metabolic viability relative to cells exposed to PLGA/PEG containing no drug. Drug doses (temozolomide–1 mmol/L; etoposide–8.5 μ mol/L) greater than the previously determined IC₅₀ concentrations were chosen to ensure sufficient drug concentrations were achieved after 24-hour burst release. Released temozolomide, etoposide, and combined temozolomide/etoposide resulted in 88.5% \pm 3.2, 50.0% \pm 1.0, and 45% \pm 2.2 metabolic viability, respectively, confirming these agents retain cytotoxic capabilities when released from PLGA/PEG formulated with 0.05% lactic acid (Fig. 2F).

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

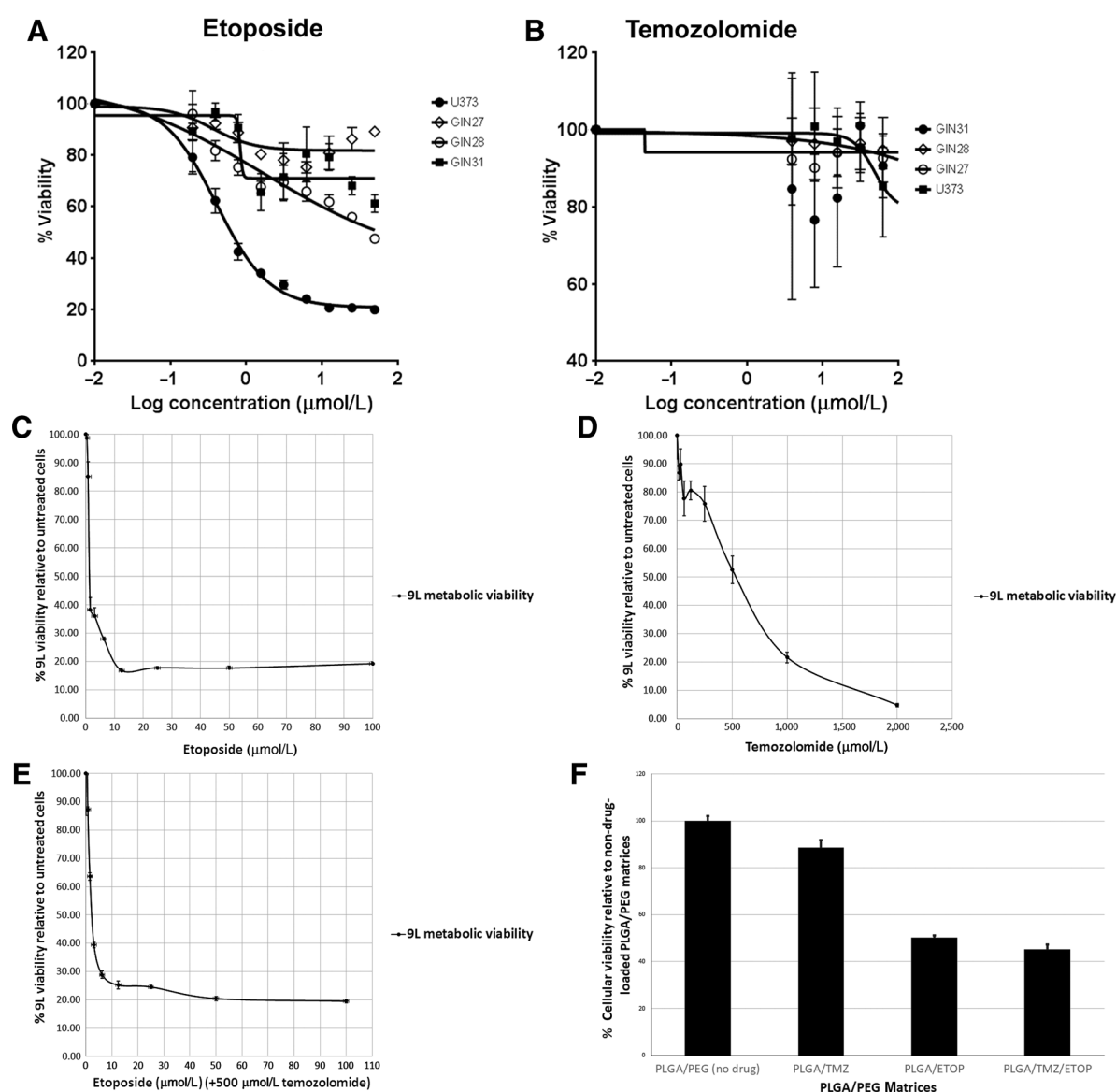
To determine safety of intracavity delivery, PLGA/PEG/temozolomide/etoposide paste was molded to the tumor cavity lining of rat brains immediately after surgical resection of 9L allograft gliomas (Fig. 3A and B), which were either implanted 5 days prior to, or concurrently with polymer paste. Previous PLGA/PEG/temozolomide/etoposide dosing to determine MTD in human GBM subcutaneous mouse xenografts, showed that 50-mg PLGA/PEG containing either 15% w/w temozolomide/50% w/w etoposide or 20% w/w temozolomide/50% w/w etoposide, was well tolerated over 102 and 76 days, respectively. A dose of 30% w/w temozolomide/50% w/w etoposide 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) 50-mg PLGA/PEG containing 10% w/w temozolomide/25% w/w etoposide; (ii) 50-mg PLGA/PEG containing 20% w/w temozolomide/50% w/w etoposide. 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 neurologic deficits, whereas no neurologic deficit was observed in PLGA/PEG/temozolomide/etoposide animals (Fig. 3C and D).

In vivo efficacy of intracavity delivered PLGA/PEG/temozolomide/etoposide in orthotopic glioma allografts

At the termination of *in vivo* tolerability studies at day 50 postsurgery and polymer implant, both PLGA/PEG containing 10% w/w temozolomide/25% w/w etoposide or 20% w/w temozolomide/50% w/w etoposide, resulted in a significant survival

**Figure 1.**

In vitro release of temozolomide (TMZ) and combined temozolomide/etoposide (ETOP) from an acidic pH formulation of PLGA/PEG paste. **A**, Scanning electron images of PLGA/PEG microparticulate paste molded into 12-mm × 6-mm-diameter cylindrical matrices, each loaded with 500-μg temozolomide 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 temozolomide and create the paste. Top, ×50; bottom, ×500. **B**, *In vitro* cumulative release of temozolomide prodrug from PLGA/PEG matrices loaded with 500 μg of drug. The release study was performed in PBS (pH 7.4) at 37°C and temozolomide quantified using LC/MS for a 10-day period, at which time point, all the drug was released. Error bars indicate the SEM from three independent matrices. **C** and **D**, Mass spectrum of a sample measured at day 1 and day 7 of the *in vitro* release period showing the conservation of the temozolomide molecular ion at 195.14 m/z (indicated by asterisks). **E**, *In vitro* dual cumulative release of temozolomide and etoposide from PLGA/PEG matrices loaded with 500 μg of each drug and containing lactic acid carrier phase, was performed in PBS (pH 7.4) at 37°C and quantified using LC/MS. Temozolomide shows a 7-day release profile where total prodrug has been released and etoposide shows a 22-day release profile. Error bars, SEM from three independent matrices.

**Figure 2.**

In vitro cytotoxicity of human and rodent high-grade glioma cells exposed to temozolomide (TMZ) and etoposide (ETOP). **A**, A panel of human GBM cell lines were exposed to a concentration range of 0.01–500 $\mu\text{mol/L}$ etoposide over 72 hours. Sensitivity to etoposide was determined by impairment of metabolic activity (PrestoBlue assay), relative to vehicle-controlled untreated cells, with an observed IC_{50} concentration of $0.4 \mu\text{mol/L} \pm 1.8$ 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 temozolomide under any drug dose, although minimal but significant impaired metabolic viability was observed at 500 $\mu\text{mol/L}$ temozolomide after 72 hours: U-373MG, 79.9% viability ± 5.5 ; GIN-27, 89.0% viability ± 3.5 ; GIN-28, 86.5% viability ± 3.02 ; GIN-31, 89.5% ± 6.1 . **A** and **B**, Error bars represent SEM 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 etoposide dose range of 0.39–100 $\mu\text{mol/L}$ over 72 hours, with an IC_{50} concentration of $<3.12 \mu\text{mol/L}$ (36.0% viability ± 3.0). **D**, Impaired 9L metabolic viability was observed within a temozolomide dose range of 15.6–2,000 $\mu\text{mol/L}$ over 72 hours, with an IC_{50} concentration of 500 $\mu\text{mol/L}$ (52.5% viability ± 4.7). **E**, To determine synergy, the IC_{50} dose of temozolomide (500 $\mu\text{mol/L}$) was added to individual wells containing etoposide at 0.39–100 $\mu\text{mol/L}$ for 72 hours, with an IC_{50} concentration of $<3.12 \mu\text{mol/L}$ (39.4% viability ± 0.9) observed. **F**, 9L cells exposed to temozolomide, etoposide, or combined temozolomide/etoposide released from PLGA/PEG matrices resulted in $88.5\% \pm 3.2$, $50.0\% \pm 1.0$ and $45\% \pm 2.2$ metabolic viability, respectively, relative to cells exposed to PLGA/PEG containing no drug.

benefit over sham surgery and *per ore* temozolomide 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 IHC on

postsacrificial brain tissue (Supplementary Figs. S3A–S3D and S4). As this was evident whether 9L allografts were implanted 5 days prior to surgery or on the day of surgery, the former tumor implantation timepoint was selected for a therapy study as this more closely mimics the clinical scenario. Evidence of both

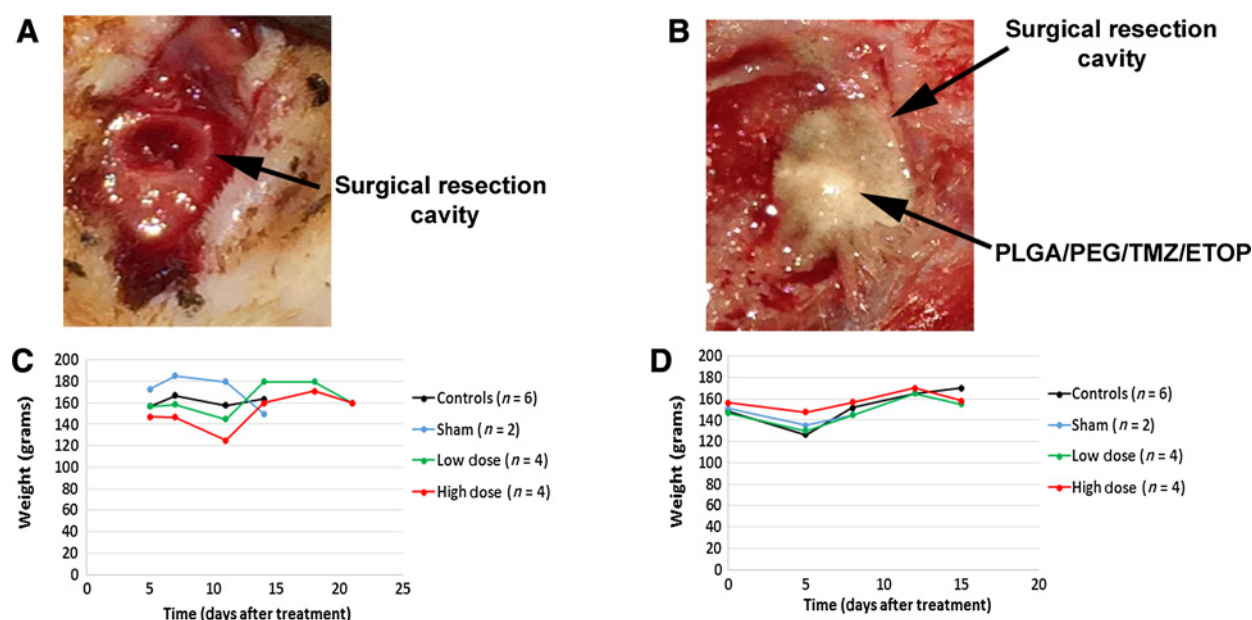


Figure 3.

In vivo tolerability of PLGA/PEG/temozolomide (TMZ)/etoposide (ETOP) in orthotopic gliomas. **A**, Rat with surgically resected 9L tumor showing surgical cavity margins. **B**, Rat with PLGA/PEG paste loaded with combined temozolomide and etoposide, molded to the resection cavity lining. **C** and **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 temozolomide (5 mg) and 25% w/w etoposide (12.5 mg); high dose, surgery/PLGA/PEG containing 20% w/w temozolomide (10 mg) and 50% w/w etoposide (25 mg; n = number of animals per arm).

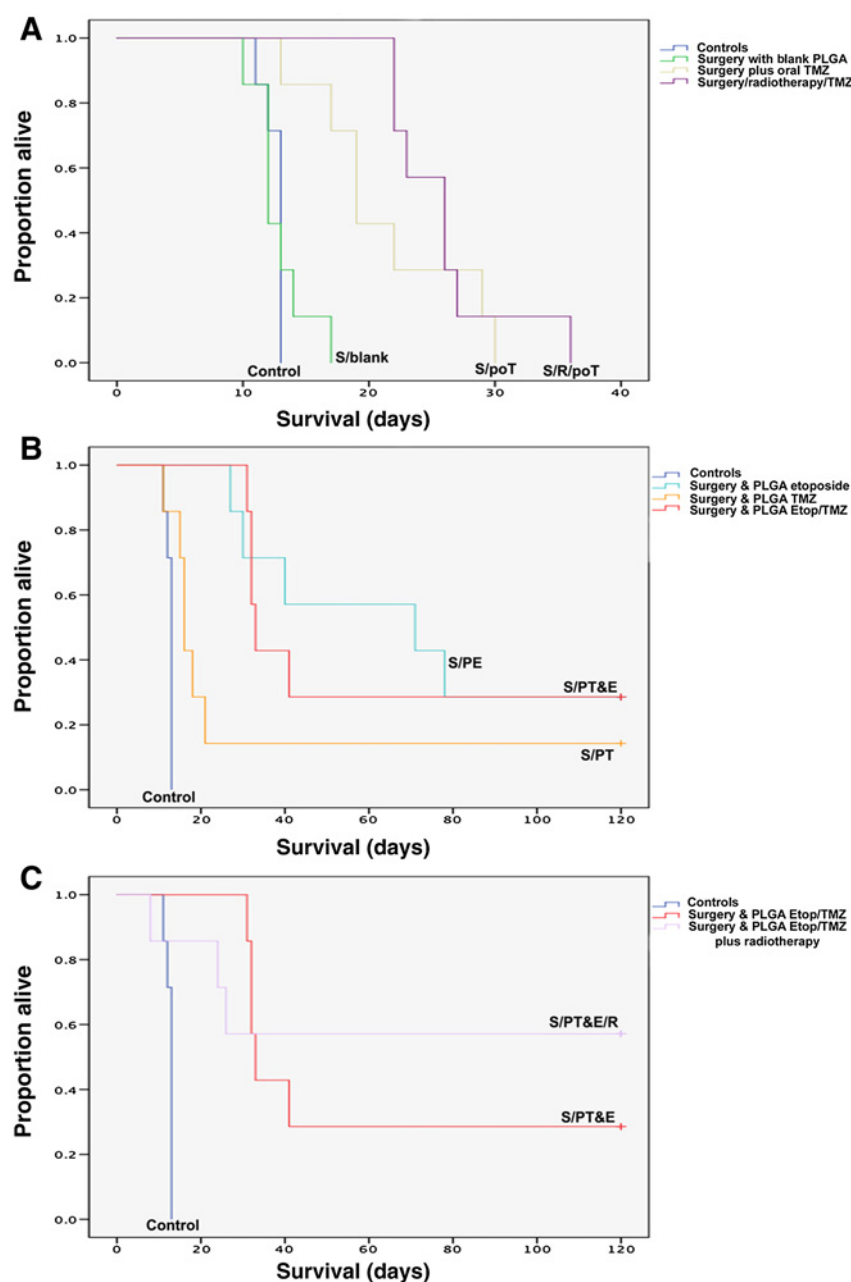
extensive infiltration in the adjacent brain parenchyma close to the primary tumor site and individual infiltrative cells in the contralateral hemisphere, in animals receiving sham surgery, confirm the aggressive invasive nature of the 9L allograft (Supplementary Fig. S3E–S3H).

To ensure statistical significance within a clinically relevant powered therapy study, $n = 7$ animals were used per treatment arm, adjuvant radiotherapy was included, and day 120 post-treatment was regarded as a measure of long-term survivors (LTS) as reported previously by us (12, 35). Intracavity delivery of PLGA/PEG/temozolomide/etoposide consistently improved the survival of tumor-bearing animals compared with control arms. Untreated animals, animals that received surgery/blank polymer, and animals that received surgery/*per orem* temozolomide (Fig. 4A; Table 1) had a median survival of 13.0, 12.0, and 19.0 days, respectively. Animals that received surgery, *per orem* temozolomide and radiotherapy (Stupp protocol) had a relatively increased median survival of 26.0 days [$P < 0.0001$ vs. controls, surgery/blank polymer (Fig. 4A) and surgery/radiotherapy (Table 1)]. No significant difference was observed between the Stupp protocol and animals that received surgery/*per orem* temozolomide (S/poT, $n = 7$; $P = 0.336$; Fig. 4A; Table 1). Animals that received either surgery/PLGA/PEG/temozolomide/etoposide or surgery/PLGA/PEG/etoposide, had an increased median survival of 33.0 and 71.0 days, respectively, compared with surgery/PLGA/PEG/temozolomide ($P < 0.0001$ vs. controls; $P < 0.0001$ vs. surgery/PLGA/PEG/temozolomide), with 28.6% LTS in each group (Fig. 4B; Table 1). Animals that received surgery/PLGA/PEG/temozolomide/etoposide with adjuvant radiotherapy had an increased mean survival of 76.8 days relative to animals that received surgery/PLGA/PEG/temozolomide/eto-

poside ($P < 0.0001$). Because of four of seven LTS in the surgery/PLGA/PEG/temozolomide/etoposide with adjuvant radiotherapy group, median survival could not be determined (Fig. 4C). A comparison of survival among all groups revealed animals receiving surgery/PLGA/PEG/temozolomide/etoposide with adjuvant radiotherapy had the greatest overall survival benefit, with 57.1% of animals deemed LTS, relative to 28.6% LTS for surgery/PLGA/PEG/temozolomide/etoposide, 28.6% LTS for surgery/PLGA/PEG/etoposide, and 14.3% LTS in animals treated with surgery and radiotherapy (Table 1).

Histologic and immunohistochemical confirmation of *in vivo* efficacy

To confirm that the observed survival benefit in PLGA/PEG treatment groups was directly due to efficacious intracavity delivery of temozolomide/etoposide, histologic and immunohistochemical analyses at the surgical margins and adjacent parenchyma were conducted on postsacrificial brains. Untreated animals (day 13), animals treated with surgery/*per orem* temozolomide (day 13), surgery/*per orem* temozolomide/radiotherapy (day 22), surgery/radiotherapy (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 (Fig. 5A–E). This finding is consistent with IHC staining for the proliferation marker Ki67, where untreated animals (day 13), animals treated with surgery/*per orem* temozolomide (day 13), surgery/*per orem* temozolomide/radiotherapy (day 36), surgery/radiotherapy (day 26), or surgery/blank PLGA/PEG (day 12), revealed high numbers of proliferative cells that have infiltrated into the surgical resection

**Figure 4.**

In vivo efficacy of interstitially delivered PLGA/PEG/temozolomide (TMZ)/etoposide (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 + radiotherapy (XRT) + *per ore*m temozolomide by gavage (Stupp standard-of-care protocol, $n = 7$); surgery + radiotherapy; surgery + 50-mg PLGA/PEG; surgery + 50-mg PLGA/PEG containing 20% w/w temozolomide and 50% w/w etoposide; surgery + 50-mg PLGA/PEG containing 20% w/w temozolomide; surgery + 50-mg PLGA/PEG containing 50% w/w etoposide; surgery + 50-mg PLGA/PEG containing 20% w/w temozolomide and 50% w/w etoposide + radiotherapy. **A**, Animals that received surgery, *per ore*m temozolomide and radiotherapy (S/R/poT; Stupp protocol) had a relatively increased median survival compared with 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 ore*m temozolomide (S/poT, $n = 7$; $P = 0.336$). **B**, While animals that received either surgery and PLGA/PEG/temozolomide/etoposide (S/P/T&E, $n = 7$), surgery, and PLGA/PEG/etoposide (S/PE, $n = 7$) or surgery and PLGA/PEG/temozolomide (S/PT, $n = 7$) had a relatively increased median survival compared with untreated animals (9L control, $n = 7$; $P < 0.0001$ for each comparison), animals that received either surgery and PLGA/PEG/temozolomide/etoposide (S/P/T&E, $n = 7$) or surgery and PLGA/PEG/etoposide (S/PE, $n = 7$), had a relatively increased median survival compared with animals that received surgery and PLGA/PEG/temozolomide (S/PT, $n = 7$; $P < 0.0001$ for each comparison). **C**, Animals that received surgery and PLGA/PEG/temozolomide/etoposide with adjuvant radiotherapy (S/P/T&E/R, $n = 7$) had an increased mean survival relative to animals that received surgery and PLGA/PEG/temozolomide/etoposide (S/P/T&E, $n = 7$; $P < 0.0001$). Animals alive at termination of experiment after 120 days postsurgery and polymer implant were deemed LTSs.

Table 1. Summary of median and mean overall survival in 9L orthotopic allografts treated with PLGA/PEG-delivered temozolomide/etoposide \pm radiotherapy

Group ($n = 7$ per group)	Group (label)	Mean ^a survival (days)	Median survival (days)	SEM	LTS	% LTS
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

NOTE: LTSs are evident in PLGA/PEG treatment arms, relative to control arms with PLGA/PEG/temozolomide/etoposide/radiotherapy resulting in the highest relative percentage (57.1%) of LTS.

Abbreviations: T, 10-mg temozolomide; E, 25-mg etoposide; S, surgery; R, irradiation (10 Gy); poT, *per ore*m temozolomide (50 mg/kg/day for 5 days).

^aEstimation is limited to the largest survival time if it is censored.

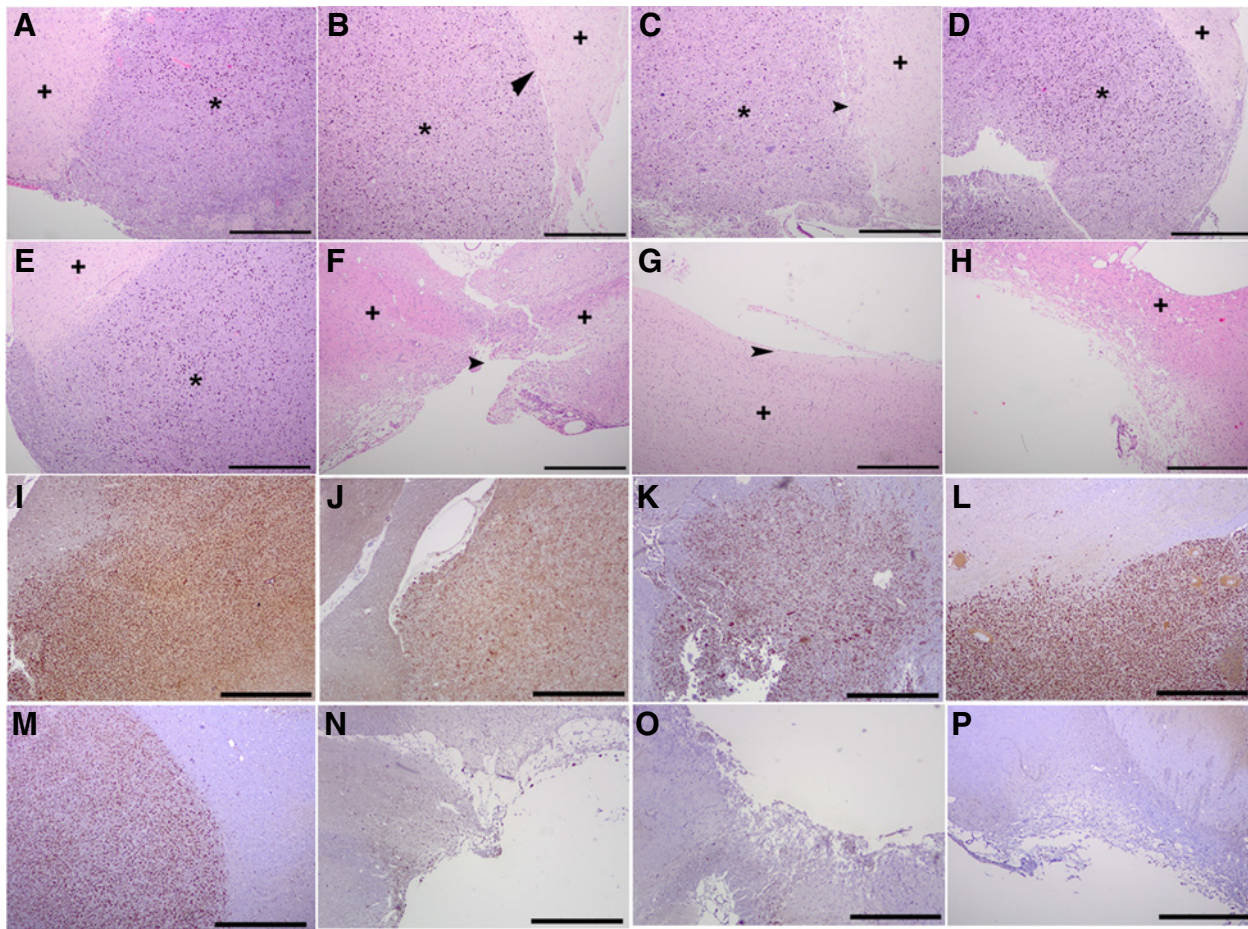


Figure 5.

Histologic and IHC confirmation of efficacy after 120 days post-PLGA/PEG/temozolomide (TMZ)/etoposide (ETOP) interstitial delivery to orthotopic 9L gliosarcomas. Hematoxylin and eosin staining: control untreated day 13 (A), surgery/*per orem* temozolomide day 13 (B), surgery/radiotherapy/*per orem* temozolomide day 22 (C), surgery/radiotherapy day 26 (D), and surgery/blank PLGA/PEG day 14 (E), 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 +). Surgery/PLGA/PEG/temozolomide/etoposide day 120 (F), surgery/PLGA/PEG/etoposide day 120 (G), and surgery/PLGA/PEG/temozolomide/etoposide/radiotherapy day 120 (H), show gliotic scarring but no recurrent tumor cells. Ki67 (proliferation marker) IHC: control untreated day 13 (I), surgery/*per orem* temozolomide day 13 (J), surgery/radiotherapy/*per orem* temozolomide day 36 (K), surgery/radiotherapy day 26 (L) and surgery/blank PLGA/PEG day 12, reveal high numbers of proliferative cells which have infiltrated into the surgical resection cavity (M). Surgery/PLGA/PEG/temozolomide/etoposide day 120 (N), surgery/PLGA/PEG/etoposide day 120 (O), and surgery/PLGA/PEG/temozolomide/etoposide/radiotherapy day 120 (P), show a surgical resection site with no visible proliferative cells. All images taken at $\times 40$. Scale bar A–H, 500 μm . "Days", days post-polymer implant.

cavity and brain parenchyma (Fig. 5I–M). In contrast, animals treated with surgery/PLGA/PEG/temozolomide/etoposide (day 120), surgery/PLGA-PEG-etoposide (day 120), and surgery/PLGA/PEG/temozolomide/etoposide/radiotherapy (day 120), show gliotic scarring but no histologic/IHC evidence of recurrent proliferative tumor cells, consistent with long-term survivorship (Fig. 5F–H, O, and P). To further visualize treatment efficacy, histologic staining on whole-brain cross-sections confirmed extensive 9L tumor regrowth and infiltration beyond the resection cavity in untreated and surgery/*per orem* temozolomide animals. In marked contrast, LTS animals representative of surgery/PLGA/PEG/temozolomide/etoposide and surgery/PLGA/PEG/temozolomide/etoposide/radiotherapy treatment groups, showed no evidence of tumor cells within and beyond the surgical resection cavity, confirming that these animals were likely disease-free (Supplementary Fig. S5).

Discussion

Despite a substantial increase in clinical trials for high-grade glioma in the postgenomic 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 noninvasive, low-intensity alternating electrical fields (tumor treating fields; TTF) in combination with temozolomide, 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; ref. 39). The difficulty of delivering therapeutic doses across the BBB remains a substantial impediment to candidate molecular therapies. An increasing appreciation of intratumor heterogeneity and subclonal divergence presents a compounding obstacle and

highlights an urgent need to consider combination therapy in next-generation clinical trials (40–42).

Intracavity 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 regrowth. 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 intracavity delivery of repurposed agents using biomaterials in preclinical orthotopic brain tumor models (43–46).

In this study, we have assessed *in vivo* efficacy of a novel intracavity delivery mode, whereby a biodegradable formulation of PLGA/PEG loaded with a combination of temozolomide/etoposide is administered as a paste that molds to the irregular contours of a tumor resection cavity, sintering *in situ* and maintaining close conformity to the cavity lining. Our data reveal a significant overall survival benefit in surgery/PLGA/PEG/temozolomide/etoposide-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 with that of BCNU/carmustine previously reported as preclinical support for the clinical translation of Gliadel and also comparable with LTS of BCNU/temozolomide 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 oversimplifying 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 overpredicting the clinical survival benefit of temozolomide/etoposide delivered by PLGA/PEG in our study, the survival advantage to a subset of patients with GBM 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 etoposide 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 etoposide was applied by intratumoral administration of catheters and not targeted to clinically relevant postresection residual disease (53). Our findings warrant clinical translation consideration to test whether subsets of patients with GBM may respond to localized etoposide therapy.

Whereas temozolomide instability has rarely been considered for local delivery studies historically, we have prepared PLGA/PEG using an organic acid-based carrier to ensure temozolomide 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 temozolomide/etoposide from PLGA/PEG. Despite surgery/PLGA/PEG/temozolomide conferring a median survival advantage over surgery/*per orem* temozolomide, including one LTS, 9L is more sensitive to etoposide. Surgery/PLGA/PEG/etoposide is comparably efficacious to surgery/PLGA/PEG/temozolomide/etoposide, consistent with comparable IC_{50} concentrations from combined etoposide/temozolomide or etoposide 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/temozolomide against subsets of GBM with varying degrees of MGMT promoter methylation or other molecular resistance mechanisms. Indeed, our formulation is applicable for next-generation temozolomide 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 etoposide/temozolomide (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 postsurgery when tumor burden is relatively minimal.

Despite using etoposide MTD determined from our previous GBM subcutaneous xenograft study (22), etoposide MTD was not reached for orthotopic dosing, indicating that dose-limiting systemic toxicities are avoided by intracavity 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/etoposide/temozolomide should be safe for clinical trial translation.

In summary, PLGA/PEG paste-mediated intracavity delivery of etoposide/temozolomide as an adjuvant to radiotherapy 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 etoposide for PLGA/PEG localized delivery and offer 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.

Disclosure of Potential Conflicts of Interest

K.M. Shakesheff reports receiving other remuneration from Locate Bio Ltd. No potential conflicts of interest were disclosed by the other authors.

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References

- Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol* 2016;131:803–20.
- Delgado-López PD, Corrales-García EM. Survival in glioblastoma: a review on the impact of treatment modalities. *Clin Transl Oncol* 2016;18:1062–71.
- Nizamutdinov D, Stock EM, Dandashi JA, Vasquez EA, Mao Y, Dayawansa S, et al. Prognostication of survival outcomes in patients diagnosed with glioblastoma. *World Neurosurg* 2018;109:e67–74.
- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987–96.
- Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin* 2017;67:7–30.
- Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen H-J, et al. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol* 2006;7:392–401.
- Lau D, Hervey-Jumper SL, Chang S, Molinaro AM, McDermott MW, Phillips JJ, et al. A prospective phase II clinical trial of 5-aminolevulinic acid to assess the correlation of intraoperative fluorescence intensity and degree of histologic cellularity during resection of high-grade gliomas. *J Neurosurg* 2016;124:1300–9.
- Calvert AE, Chalastanis A, Wu Y, Hurley LA, Kouri FM, Bi Y, et al. Cancer-Associated IDH1 promotes growth and resistance to targeted therapies in the absence of mutation. *Cell Rep* 2017;19:1858–73.
- Quinn JA, Jiang SX, Reardon DA, Desjardins A, Vredenburgh JJ, Rich JN, et al. Phase II trial of temozolomide plus O 6 -benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma. *J Clin Oncol* 2009;27:1262–7.
- de Gooijer MC, de Vries NA, Buckle T, Buil LCM, Beijnen JH, Boogerd W, et al. Improved brain penetration and antitumor efficacy of temozolomide by inhibition of ABCB1 and ABCG2. *Neoplasia* 2018;20:710–20.
- Ostermann S, Csajka C, Buclin T, Leyvraz S, Lejeune F, Decosterd LA, et al. Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients. *Clin Cancer Res* 2004;10:3728–36.
- Brem S, Tyler B, Li K, Pradilla G, Legnani F, Caplan J, et al. Local delivery of temozolomide by biodegradable polymers is superior to oral administration in a rodent glioma model. *Cancer Chemother Pharmacol* 2007;60:643–50.
- Ganipineni LP, Danhier F, Pr  at V. REVIEW: Drug delivery challenges and future of chemotherapeutic nanomedicine for glioblastoma treatment. *J Control Release* 2018;281:42–57.
- Brem H, Piantadosi S, Burger PC, Walker M, Selker R, Vick NA, et al. Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-brain Tumor Treatment Group. *Lancet* 1995;345:1008–12.
- Westphal M, Hilt DC, Bortey E, Delavault P, Olivares R, Warnke PC, et al. A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. *Neuro Oncol* 2003;5:79–88.
- Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med* 2014;370:699–708.
- Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy–temozolomide for newly diagnosed glioblastoma. *N Engl J Med* 2014;370:709–22.
- Rahman C V, Smith SJ, Morgan PS, Langmack KA, Clarke PA, Ritchie AA, et al. Adjuvant chemotherapy for brain tumors delivered via a novel intracavity moldable polymer matrix. *PLoS One* 2013;8:e77435.
- Rahman CV, Kuhn G, White LJ, Kirby GTS, Varghese OP, McLaren JS, et al. PLGA/PEG-hydrogel composite scaffolds with controllable mechanical properties. *J Biomed Mater Res B Appl Biomater* 2013;101:648–55.
- Vellimana AK, Recinos VR, Hwang L, Fowers KD, Li KW, Zhang Y, et al. Combination of paclitaxel thermal gel depot with temozolomide and radiotherapy significantly prolongs survival in an experimental rodent glioma model. *J Neurooncol* 2013;111:229–36.
- Gerber DE, Gallia GL, Tyler BM, Eberhart CG, Royer G, Grossman SA. A novel polymer gel for the delivery of local therapies to intracranial tumors: *in vivo* safety evaluation. *J Biomed Mater Res A* 2011;99:479–84.
- Smith SJ, Rahman CV, Clarke Pa, Ritchie aa, Gould TW, Ward JH, et al. Surgical delivery of drug releasing poly(lactic-co-glycolic acid)/poly(ethylene glycol) paste with *in vivo* effects against glioblastoma. *Ann R Coll Surg Engl* 2014;96:495–501.
- Reardon DA, Desjardins A, Vredenburgh JJ, Gururangan S, Sampson JH, Sathornsumetee S, et al. Metronomic chemotherapy with daily, oral etoposide plus bevacizumab for recurrent malignant glioma: a phase II study. *Br J Cancer* 2009;101:1986–94.
- Aoki T, Mizutani T, Nojima K, Takagi T, Okumura R, Yuba Y, et al. Phase II study of ifosfamide, carboplatin, and etoposide in patients with a first recurrence of glioblastoma multiforme. *J Neurosurg* 2010;112:50–6.
- Kim H, Likhari P, Parker D, Statkevich P, Marco A, Lin CC, et al. High-performance liquid chromatographic analysis and stability of anti-tumor agent temozolomide in human plasma. *J Pharm Biomed Anal* 2001;24:461–8.
- Diez BD, Statkevich P, Zhu Y, Abutarif MA, Xuan F, Kantesaria B, et al. Evaluation of the exposure equivalence of oral versus intravenous temozolomide. *Cancer Chemother Pharmacol* 2010;65:727–34.
- Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C. Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. *Cancer Treat Rev* 1997;23:35–61.
- Masi BC, Tyler BM, Bow H, Wicks RT, Xue Y, Brem H, et al. Intracranial MEMS based temozolomide delivery in a 9L rat gliosarcoma model. *Biomaterials* 2012;33:5768–75.
- Sonabend AM, Carminucci AS, Amendolara B, Bansal M, Leung R, Lei L, et al. Convection-enhanced delivery of etoposide is effective against murine proneural glioblastoma. *Neuro Oncol* 2014;16:1210–9.
- Kuo YC, Chen YC. Targeting delivery of etoposide to inhibit the growth of human glioblastoma multiforme using lactoferrin- and folic acid-grafted poly(lactide-co-glycolide) nanoparticles. *Int J Pharm* 2015;479:138–49.

31. Lamprecht A, Benoit JP. Etoposide nanocarriers suppress glioma cell growth by intracellular drug delivery and simultaneous P-glycoprotein inhibition. *J Control Release* 2006;112:208–13.
32. Callewaert M, Dukic S, Van Gulick L, Vittier M, Gafa V, Andry MC, et al. Etoposide encapsulation in surface-modified poly(lactide-co-glycolide) nanoparticles strongly enhances glioma antitumor efficiency. *J Biomed Mater Res - Part A* 2013;101 A:1319–27.
33. Chou T-C. Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res* 2010;70:440–6.
34. Scott AW, Tyler BM, Masi BC, Upadhyay UM, Patta YR, Grossman R, et al. Intracranial microcapsule drug delivery device for the treatment of an experimental gliosarcoma model. *Biomaterials* 2011;32:2532–9.
35. Wicks RT, Azadi J, Mangraviti A, Zhang I, Hwang L, Joshi A, et al. Local delivery of cancer-cell glycolytic inhibitors in high-grade glioma. *Neuro Oncol* 2014;17:70–80.
36. Brennan CW, Verhaak RGW, McKenna A, Campos B, Nushmeh H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell* 2013;155:462–77.
37. Ceccarelli M, Barthel FP, Malta TM, Sabedot TS, Salama SR, Murray BA, et al. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. *Cell* 2016;164:550–63.
38. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010;17:98–110.
39. Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA, et al. Maintenance therapy with tumor-treating fields plus temozolomide vs temozolomide alone for glioblastoma: a randomized clinical trial. *JAMA* 2015;314:2535–43.
40. Sottoriva A, Spiteri I, Piccirillo SGM, Touloumis A, Collins VP, Marioni JC, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A* 2013;110:4009–14.
41. Eskilsson E, Rosland GV, Talasila KM, Knappskog S, Keunen O, Sottoriva A, et al. EGFRvIII mutations can emerge as late and heterogeneous events in glioblastoma development and promote angiogenesis through Src activation. *Neuro Oncol* 2016;18:1644–55.
42. Watts C, Piccirillo S, Spiteri I, Sottoriva A, Touloumis A, Marioni J, et al. Complex evolutionary dynamics generates genetic diversity and intratumor heterogeneity in individual patients with glioblastoma. *Neuro Oncol* 2014;16:iii7.
43. Zhao M, Danhier F, Bastiancich C, Joudiou N, Ganipineni LP, Tsakiris N, et al. Post-resection treatment of glioblastoma with an injectable nanomedicine-loaded photopolymerizable hydrogel induces long-term survival. *Int J Pharm* 2018;548:522–9.
44. Bastiancich C, Bianco J, Vanvarenberg K, Ucakar B, Joudiou N, Gallez B, et al. Injectable nanomedicine hydrogel for local chemotherapy of glioblastoma after surgical resection. *J Control Release* 2017;264:45–54.
45. Okolie O, Irvin DM, Bago JR, Sheets K, Satterlee A, Carey-ewend AG, et al. Intra-cavity stem cell therapy inhibits tumor progression in a novel murine model of medulloblastoma surgical resection. *PLoS One* 2018;13:0198596.
46. Shankar GM, Kirtane AR, Miller JJ, Mazdiyasni H, Rogner J, Tai T, et al. Genotype-targeted local therapy of glioma. *Proc Natl Acad Sci USA* 2018;114:E8388–94.
47. Tamargo RJ, Myseros JS, Epstein JI, Yang MB, Chasin M, Brem H. Interstitial Chemotherapy of the 9L gliosarcoma: controlled release polymers for drug delivery in the brain. *Cancer Res* 1993;53:329–33.
48. Shapira-Furman T, Serra R, Gorelick N, Doglioli M, Tagliaferri V, Cecia A, et al. Biodegradable wafers releasing temozolomide and carmustine for the treatment of brain cancer. *J Control Release* 2019;295:93–101.
49. Sipos EP, Tyler B, Piantadosi S, Burger PC, Brem H. Optimizing interstitial delivery of BCNU from controlled release polymers for the treatment of brain tumors. *Cancer Chemother Pharmacol* 1997;39:383–9.
50. Valtonen S, Timonen U, Toivanen P, Kalimo H, Kivipelto L, Heiskanen O, et al. Interstitial chemotherapy with carmustine-loaded polymers for high-grade gliomas: a randomized double-blind study. *Neurosurgery* 1997;41:44–8.
51. Chowdhary SA, Ryken T, Newton HB. Survival outcomes and safety of carmustine wafers in the treatment of high-grade gliomas: a meta-analysis. *J Neurooncol* 2015;122:367–82.
52. Qi Z, Xing W, Shao C, Yang C, Wang Z. The role of Gliadel wafers in the treatment of newly diagnosed GBM: a meta-analysis. *Drug Des Devel Ther* 2015;9:3341.
53. Sonabend AM, Carminucci AS, Amendolara B, Bansal M, Leung R, Lei L, et al. Convection-enhanced delivery of etoposide is effective against murine proneural glioblastoma. *Neuro Oncol* 2014;16:1210–9.
54. Zhang J, Stevens MFG, Hummersone M, Madhusudan S, Laughton Ca, Bradshaw TD. Certain imidazotetrazines escape O6-methylguanine-DNA methyltransferase and mismatch repair. *Oncology* 2011;80:195–207.
55. Zhang J, Hummersone M, Matthews CS, Stevens MFG, Bradshaw TD. N3-substituted temozolomide analogs overcome methylguanine-DNA methyltransferase and mismatch repair precipitating apoptotic and autophagic cancer cell death. *Oncol* 2015;88:28–48.
56. Anderson S. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Adv Drug Deliv Rev* 1997;28:5–24.
57. Langer R. Drug delivery and targeting. *Nature* 1998;392:5–10.
58. Bjugstad KB, Redmond DE, Lampe KJ, Kern DS, Sladek JR, Mahoney MJ. Biocompatibility of PEG-based hydrogels in primate brain. *Cell Transplant* 2008;17:409–15.
59. Emerich DF, Tracy MA, Ward KL, Figueiredo M, Qian R, Henschel C, et al. Biocompatibility of poly (DL-lactide-co-glycolide) microspheres implanted into the brain. *Cell Transplant* 1999;8:47–58.