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Combined intracranial Acriflavine, temozolomide and radiation extends survival in a rat glioma model

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ABSTRACT

Glioblastomas have been historically difficult to treat with poor long-term survival. With novel strategies focused on targeting hypoxia-inducible factor (HIF) regulatory pathways, recent evidence has shown that Acriflavine (ACF) can effectively target glioma invasiveness and recurrence. However, local delivery of ACF and its combinatory effects with Temozolomide (TMZ) and radiation therapy (XRT) have not yet been optimized. In this study we test a novel polymeric matrix that can gradually release ACF at the tumor bed site in combination with systemic TMZ and XRT. *In vitro* cytotoxicity assays of ACF in combination with TMZ and XRT were performed on rodent and human cell lines with CCK-8 and flow cytometry. *In vitro* drug release was measured and intracranial safety was assessed in tumor-free animals. Finally, efficacy was assessed in an intracranial gliosarcoma model and combination therapy with TMZ and XRT evaluated. Combination therapy of ACF, TMZ, and XRT was able to reduce cell viability and induce apoptosis in glioma cells. *In vitro* and *in vivo* release of ACF was measured in benchtop and animal models. Efficacy was established in an *in vivo* gliosarcoma model in which intracranial ACF ($p < 0.01$) significantly improved median survival and the combination therapy of ACF, TMZ and XRT ($p < 0.01$) significantly improved median survival and led to long-term survival (LTS). We provide evidence that ACF, combined with TMZ and XRT, led to LTS in an intracranial model of rat gliosarcoma. These findings, in combination with the use of a novel polymeric matrix that allows more gradual drug delivery, constitute a first step in the translation of this novel strategy to human use.

1. Introduction

About ~100,000 diffuse gliomas are diagnosed every year worldwide, and glioblastoma (GBM) represents approximately 75% of the total (annual incidence rate of 3.2/100,000 population and median age at diagnosis of 64 years)[1]. With a median survival of 14 to 17 months, this malignant tumor of the central nervous system has marginally responded to new therapeutics introduced in the last decades[2]. Furthermore, GBM accounts for around 15% of total brain tumors, constituting 47.7% of those with malignant features[1]. Currently, most patients diagnosed with GBM are offered a combination of radiation therapy (XRT) and systemic Temozolomide (TMZ) after tumor resection [3], with adjuvant interstitial chemotherapy (Gliadel®) administered in the surgical resection cavity to improve local tumor control. The Stupp protocol, XRT and concomitant systemic TMZ, despite significantly improving the median survival, has nonetheless not yet achieved long-term survival (LTS) for this challenging population, with several different avenues of research having been attempted in the past years,

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both locally and systemically[3–5].

Among the numerous avenues of research pursued, a number of studies and clinical trials have highlighted the importance of blood supply and tumor neovascularization for GBM growth and migrating capacity[6]. The expression of hypoxia-related genes and proteins seems to account for GBM's migrating capacity[7], and resistance to apoptosis and chemo/radiation therapy[7,8]. In particular, hypoxia-inducible factor 1 (HIF-1) appears to play a crucial role in inducing neoangiogenesis and switching cell metabolism to glycolysis, as well as promoting cell survival and oncogene signaling in hypoxic conditions[9].

In a previously published study we showed that HIF-1 targeting indirectly reduces the tumor blood supply, affecting the growth of GBM. This led to prolonged survival in a rodent model of 9L gliosarcoma after local intracranial administration of ACF-loaded polymeric wafers [10]. In the current study we hypothesized that ACF, a highly hydrophilic compound not readily able to cross the blood brain barrier, can be delivered safely in a synergistic fashion with other treatments for high-grade gliomas. By combining local ACF delivery with systemic radiation and TMZ we tested the hypothesis that ACF in combination with a current clinical treatment regimen is able to achieve significantly more LTS compared to either ACF or TMZ-XRT alone in tumor-bearing rats.

Local delivery of chemotherapeutics has been at the center of GBM research for the last decades, with the initial pre-clinical development and FDA approval of Gliadel® wafers for recurrent and primary GBM, and more recently a wave of novel wafer- and nanoparticle-based interstitial therapies[4,5,11–14]. Gliadel® is a biodegradable wafer of poly[bis(p-carboxyphenoxy propane) sebacic acid] (pCPP:SA) impregnated with the anti-cancer compound carmustine (BCNU), a previously approved systemic chemotherapeutic agent for GBM therapy. It has been used extensively in primary and recurrent GBM, and has paved the way for several other studies delivering other drug formulations[15]. While several of these studies have tried to optimize the combinatory effect of local and systemic therapeutics, many have focused on delivering compounds that would not otherwise be able to cross the blood–brain barrier (BBB). The number, variety, and chemical composition of chemotherapeutics that can be delivered via the pCPP:SA polymeric matrix has been limited by the lipophilicity and "sink effect" of the polymer. Additionally, the burst release has proven even more marked with hydrophilic drugs like ACF. In the current study we employed a novel polymeric matrix with unique anti-hydrolysis capacity, capable of gradually releasing ACF to the tumor bed, minimizing the sink effect, and decreasing possible CNS toxicities. We hypothesized that the local delivery of ACF from this novel wafer in combination with systemic TMZ and XRT will prolong survival in rodent glioma models when compared to pCPP:SA ACF-loaded wafers or to orally administered TMZ with concomitant XRT.

2. Materials and methods

2.1. Cell lines

The 9L rat gliosarcoma cell line (Brain Tumor Research Center, UCSF, CA, USA) was used for *in vitro* and *in vivo* experiments in F344 rats. Cells were maintained in culture with Dulbecco's Modified Eagle Medium (Lonza, Portsmouth, NH, USA) supplemented with 10% heat-inactivated Bovine Serum (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown at 37 °C in 5% CO2-humidified incubators (Thermo-Fisher Scientific, Waltham, MA, USA), and subcultured three times a week.

2.2. Materials

PLGA 75:25 MW 14 kDa was purchased from PURAC Biomaterials (Gorinchem, Netherlands). Acriflavine HCl and mannitol were purchased from Sigma Aldrich (Rehovot, Israel). Solvents were purchased from Biolab (Jerusalem, Israel).

2.3. Cell viability assay

Cells were seeded at a density of $2x10^3$/well in a 96-well plate (Corning, New York, NY, USA) and treated with various concentrations of ACF, ACF-TMZ and ACF-TMZ-XRT. After 48 h, Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to assess the cytotoxic effect of each combination, compared to DMSO-normalized control cells.

2.4. Apoptosis assay

Apoptosis and necrosis were detected using an Apoptosis kit (PI-AnnexinV/Dead Cell Apoptosis Kit, Invitrogen, Life Technologies) and cells were seeded at a density of $5.0x10^4$ cells/well, and treated with ACF, ACF-TMZ and ACF-TMZ-XRT. Cells were trypsinized and harvested, washed with PBS, incubated with AnnexinV Ab and PI (Invitrogen, Carlsbad, CA, USA), and analyzed with BD FACS Celesta (BD Biosciences; Becton, Dickinson and Company, San Jose, CA, USA), and data processed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

3. Preparation of ACF biodegradable polymer wafers

3.1. Coating of ACF by multiple layers of PLGA

ACF (250 mg) was added to a solution of PLGA (125 mg) in dichloromethane (DCM, 0.2 mL) and mixed well until the solvent evaporated. The formulation was coated by a second layer of PLGA by adding the residual powder to a new solution of PLGA (125 mg) in DCM (0.15 mL) to produce a formulation containing 50% w/w ACF (Fig. 1). Successive layers of PLGA were added in a similar fashion to produce ACF formulations with 3, 4 or 5 layers of PLGA at 40%, 30% or 20% ACF w/w. Following addition of the final PLGA layer, the mixture was mixed by spatula until most of the solvent evaporated. Thereafter, heptane (4 mL) was added and mixed well for 4 h by vortex. Solvents were decanted and replaced by 4 mL heptane. The mixture was mixed at room temperature overnight, solvent was decanted and the residual powder was dried under vacuum.

3.2. Blank formulation

Blank formulations were prepared as described for ACF 20% w/w, with Mannitol instead of ACF.

4. *In vitro* and *in vivo* release of ACF from biodegradable polymer wafers

4.1. In vitro release studies

10 mg of each formulation was compressed into 3 mm diameter wafer and inserted to a glass vial containing 8 mL of 10 mM phosphate-buffered saline at pH 7.4. The *in vitro* release study was performed in triplicate for all formulations at 37 °C with constant mixing over the course of 28 days. Buffer was replaced at regular intervals throughout the trial.

The ACF content in samples was analyzed by UV Absorbance at 260 nm, after obtaining a calibration curve with $R^2 = 0.99$ in the range of 0.5–20 ug/ml using Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, New Jersey, USA).

4.2. Energy dispersive X-ray analysis (EDX)

The EDX instrument with Scanning Electron Microscopy (Quanta 200, FEI Company) was equipped with an EDX detector. Analyses were performed by applying particles of ACF-PLGA formulations with 20, 30, 40, and 50% w/w drug content which were secured on aluminum stubs using a conductive double-sided tape and were then sputter coated with palladium at 40 mV for 40 s prior to analysis. The specimens were

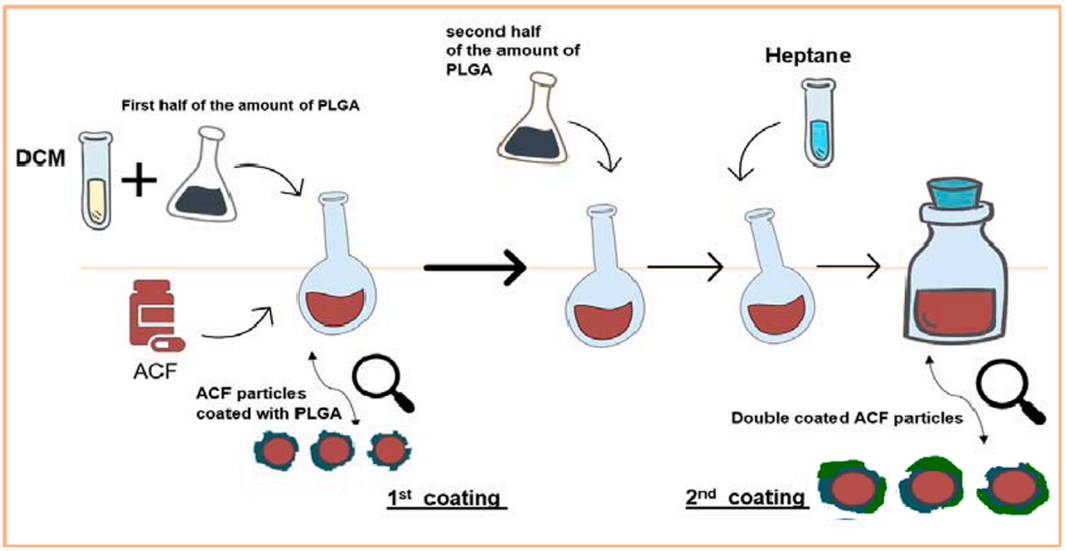

Fig. 1. Visual representation of the two PLGA coatings of ACF 50 %w/w.

detected under vacuum (upper limit of 6×10^{-6} mbar) using EDX detector.

4.3. Spectroscopy

Fourier transform infrared spectroscopy (FTIR) (Nicolet iS10, Thermo Scientific, MA, USA) was used to determine and compare the functional groups of Acriflavine, PLGA and formulations thereof.

4.4. Animal studies

Animal experiments were conducted in accordance with the guidelines of the Johns Hopkins University Animal Care and Use Committee (ACUC). For the *in vivo* studies an orthotopic 9L gliosarcoma model was implanted using tumor pieces in 82 F344 rats (50 rats for the first survival study, 32 rats for the second). Rats were anesthetized with 0.4 mL of intraperitoneal (IP) ketamine hydrochloride, 75 mg/mL (Ketathesia, Butler Animal Health Supply; Dublin, OH); xylazine 7.5 mg/mL (Lloyd Laboratories; Shenandoah, Iowa), and 14.25% ethyl alcohol in 0.9% NaCl 3 mL/kg. The head was shaved and prepared with Prepodyne solution (West Penetone, Montreal, Canada). Following a midline scalp incision, the galea was swept laterally and a left parietal 3 mm burr-hole was placed 3 mm lateral and 3 mm posterior to bregma. A durotomy was then performed under surgical microscope magnification, and a small cortical area was resected to create enough space for a 2 mm^3 tumor allograft. The skin was closed in a sterile fashion using surgical staples.

For the first *in vivo* experiment, animals were randomized into six groups based on their treatment plan. Ten rats were allocated to the control cohort, while the other groups received eight animals each. A second cohort was implanted intracranially with empty polymeric wafers, to control for local and systemic toxicities secondary to the matrix. A third group received 20% ACF-loaded polymers on day 0, immediately after 9L tumor implantation, similar to our previous experiments. A fourth group was implanted with 20% ACF polymers on day 5, a fifth group was treated with oral temozolomide (oTMZ, dose of 50 mg/kg) for 5 days (daily oral gavage from days 5 to 9) and concurrent radiation (XRT, 10 Gy single-dose on day 5), and a sixth group was treated with a combination of local 20% ACF polymer, oTMZ (dose of 50 mg/kg), and radiation (10 Gy single-dose at day 5). A second *in vivo* experiment was performed on rats implanted with 9L tumors as described to validate the initial findings, comparing untreated control animals (n = 8), against experimental groups treated on day five with either 20% ACF polymers (n = 8), oTMZ-XRT (daily oral gavage from days 5 to 9) (n = 8), or combination ACF polymers-oTMZ-XRT (n = 8). The animals were allowed to awaken, then released to their cages and received lab chow and water ad libitum. All the rats were evaluated post-operatively and monitored daily for signs of neurotoxicity and generalized toxicity (failure to thrive, neurologic deficits, lethargy, ataxia, paralysis, hunched posture per protocol. Animals were sacrificed when a humane survival endpoint was reached). Euthanasia was carried out following ACUC's guidelines. Overall, median and LTS were assessed, and autopsies performed at the end of the studies. Brains were removed, processed, and preserved in 10% formalin for histological analysis.

4.5. Histological analysis

Brains were harvested after animal perfusion and fixed in 4% paraformaldehyde for 1 day, samples were then moved to a 10% formalin solution for long-term preservation and paraffin-embedded by the Johns Hopkins University Histopathology Core for final staining. Histology section slides were prepared at 10 μm with a Leica CM1905 microtome (Leica, Wetzlar, Germany). Brains were then stained with hematoxylin-eosin (Sigma-Aldrich, St. Louis, MO, USA) and evaluated for tumor growth/invasion, signs of toxicity, and tissue damage.

4.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism Software (Version 6.0, GrapPad Software, San Diego, CA). One-way ANOVA with Bonferroni or Tukey post-tests or a non-parametric Kruskal-Wallis test were used, and graphs represent the mean ± SEM. For survival experiments, survival was represented with a Kaplan-Meier estimator, and log-rank analysis was used to establish statistical significance.

5. Results

5.1. Combination therapy reduces cell viability and induces apoptosis in glioma cells

9L gliosarcoma cell viability was significantly reduced 48 h after treatment with the combination of ACF and XRT-TMZ as assessed by CCK-8 assay compared to control cells (p-value 0.0002). Interestingly, the combination of ACF with XRT-TMZ achieved a superior result compared to each of the respective treatment arms separately ($p < 0.01$). This result reproduces previous findings from our group, showing the synergistic effect of ACF in combination with standard

chemoradiation regimens (Fig. 2). Similarly, AnnV-PI levels were significantly increased in the combination group compared to the separate treatment arms and the control group (Fig. 2).

5.2. ACF biodegradable polymer wafer characteristics and in vitro and in vivo release of ACF

Implantable wafers of ACF coated with PLGA were prepared by repeated addition of ACF particles into polymer solution followed by evaporation of the solvents. The percentage of ACF in wafers ranged from 50% to 20% w/w with increasing polymer coating steps (Table 1). FTIR and EDX were used for evaluation of the coating process as well as characterization of the resultant formulation. ACF has a characteristic IR C = C or C = N stretch at 1639 cm^{-1} while PLGA displays a band representative of ester carbonyl bond at 1749 cm^{-1}. Increased PLGA relative to ACF may be observed by relative increase of intensity of characteristic peaks (Fig. 3).

EDX is an X-ray technique used to identify the elemental composition of a substance's surface materials. Here, we employed this technique to determine successful coating of PLGA over ACF molecules. As shown in Table 2, the % detection of the nitrogen (N) atoms in ACF on the surface of polymers decreased with each coating procedure due to increased coating by PLGA, which has no N content itself. The % N detected in ACF with only one coating was 10.11%, which decreased to 6.72% after two coatings and further to 5.25% after 3 coatings. The opposite trend may be observed for % O, which increases with each O-rich PLGA coating.

The *in vitro* release profile of the 4 different biodegradable ACF-PLGA polymer formulations is presented in Fig. 4. Formulation [A], containing 50% ACF after 2 PLGA coatings, released 70% of the drug after one week and then plateaued for the following three weeks. Formulations [B] (40% ACF, 3 coatings) and [C] (30% ACF, 4 coatings) both released 78% of the drug within one week. The 40% ACF polymer reached 90% release after 28 days and the 30% ACF polymer released 82% of the drug in the same time period. Formulation [D] with 20% ACF released the drug the slowest, with 70% released in 28 days, demonstrating a more sustained release profile. Only formulation [D] did not display a burst release. This formulation was therefore selected for animal studies (Fig. 5).

5.3. Local Acriflavine release from biodegradable polymeric wafers

In vivo release of ACF from biodegradable polymers with incremental w:w ratios was performed in naïve F344 rats (that is, without intracranial 9L tumors) in a preliminary safety phase of the study. Polymer discs loaded with ACF fractions of 0–10–20–30–40–50% were implanted in the left parietal cortex of rats under surgical microscopic guidance.

Table 1
Biodegradable ACF Polymer Formulations A – D by number of PLGA coating layers and final % ACF content, w/w. ACF was coated by PLGA in two or more layers at various w/w values.

	No. of PLGA coating layers	% ACF (w/w)
Formulation A	2	50
Formulation B	3	40
Formulation C	4	30
Formulation D	5	20

Animals were observed for signs and symptoms of neuro- and general toxicities, and weighed daily. On Day 150 the animals were euthanized and the brains collected and analyzed under microscopy for signs of necrosis and inflammatory reaction to the polymeric discs. The 20% ACF:polymer ratio was chosen for the efficacy studies, given the safety displayed in this group and minimal weight loss observed immediately post-implantation and until day 150, when the study was terminated.

5.4. Combination ACF-TMX-XRT therapy prolongs LTS in vivo

We investigated the safety and efficacy of ACF-releasing polymeric wafers *in vivo* in a 9L gliosarcoma model, with concurrent (day 0) and delayed (day 5) treatment (Fig. 6). Polymeric discs were loaded with a ratio of 20% ACF:polymer, preserved at −80 °C and either implanted immediately after the 9L tumor (day 0) or in a delayed fashion, 5 days after tumor seeding (day 5). To optimize reproducibility and inter-trial reliability, we chose to repeat the experiment using the same methods and drug concentrations/polymeric wafers, tumor model and radiation parameters. In the first trial, untreated control animals achieved a median survival of 14 days post-tumor implantation, similar to that of F344 rats implanted with blank polymeric wafer (13 days, $p = 0.73$). Importantly, animals treated with ACF-containing wafers on day 0 showed significantly prolonged median, overall and LTS compared to untreated control rats ($p < 0.01$, 7/8 ACF-treated animals reached LTS at day 120), similar to our previously published results. Among the animals treated on day 5 post-tumor implantation, those implanted with 20% ACF polymer reached a median survival of 22.5 days ($p < 0.01$, compared to controls), while TMZ-XRT-treated rats achieved 33 days ($p < 0.01$) and combination 20% ACF-TMZ-XRT had a median survival of 29.5 days ($p < 0.01$). The median survival was not statistically different among these groups, however, LTS was achieved by 2 animals in the combination group (25% of the total, 2/8). In the second *in vivo* experiment the control group (n = 8) achieved a median survival of 11 days, while the 20% ACF polymer, TMZ-XRT, and combination ACF-TMZ-XRT groups reached 24 ($p < 0.01$), 27 ($p < 0.01$), and 32 days

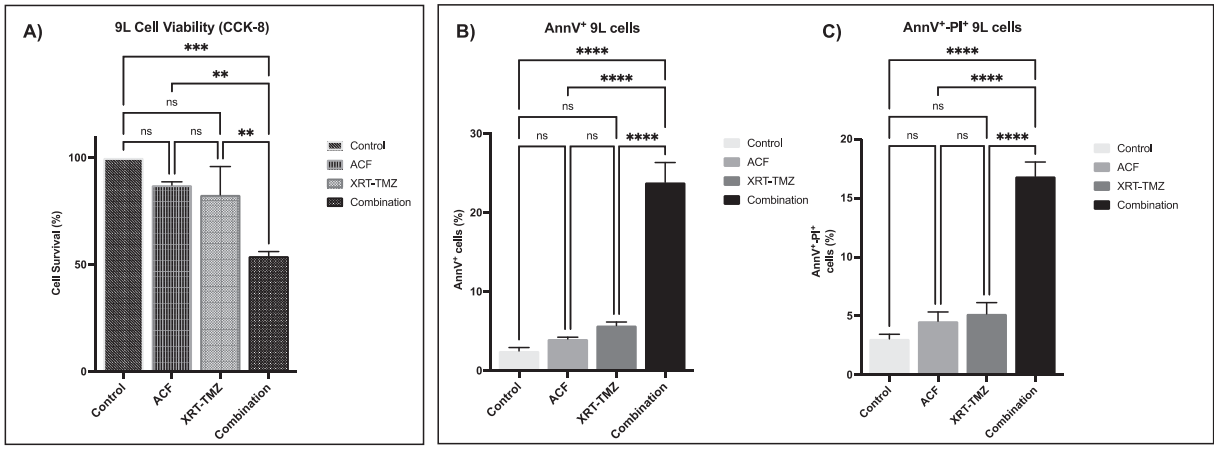

Fig. 2. Cytotoxic effects of Acriflavine in 9L gliosarcoma cells. A) Cell survival after treatment of 9L gliosarcoma cells was assessed with CCK-8 assay. **B-C)** Cytotoxic effect of ACF, TMZ and radiation on cell cultures of 9L gliosarcoma. $AnnV^+$ and (C) $AnnV^+$-PI^+ cells were assessed with flow cytometry 48 h post-treatment.

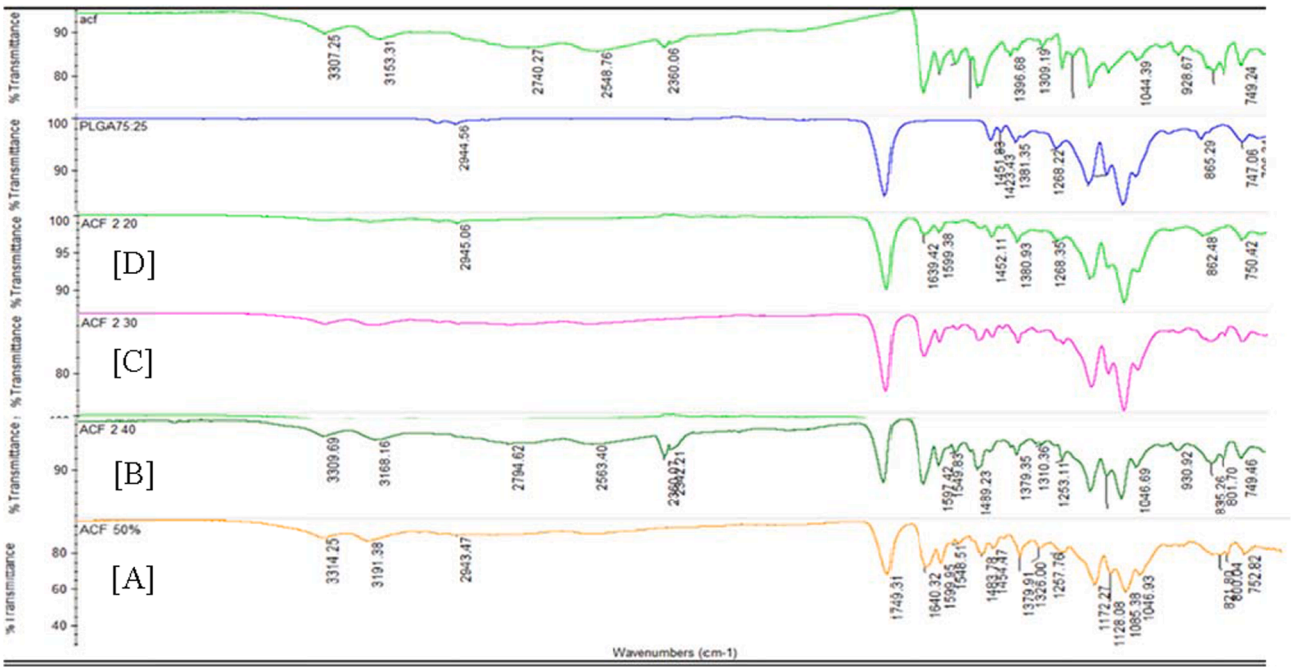

Fig. 3. FTIR spectra of ACF, PLGA 75/25 and formulations [A]-[D]. Characteristic peak of ACF is seen at 1639 cm^{-1} and for PLGA at 1739 cm^{-1}.

Table 2
% C, N and O in all formulations. As the ACF percent decreases the N% deceases and O% increases.

Element	50% ACF 1st coating	50% ACF 2nd coating [A]	40% ACF [B]	30% ACF [C]	20% ACF [D]
	Atomic%	Atomic%	Atomic%	Atomic%	Atomic%
C	76.94	63.7	69.39	74.68	64.5
N	10.11	6.72	5.25	4.18	–
O	20.11	24.82	26.34	28.14	37.54

($p < 0.01$), respectively. In this case 4 rats in the ACF-TMZ-XRT group (~50%, 4/8 survived until day 150 post-tumor implantation, and were deemed LTS. Aside from the full combination therapy group, no animals from the other experimental treatment arms reached LTS, suggesting an important benefit conferred by the combination of multiple chemo- and radio-therapeutic approaches. Notably, the LTS did not show behavioral signs of local or generalized toxicity, and were free from neurological deficits from day one post-surgery through the entire duration of the experiment.

5.5. Histological analysis

To confirm the survival data and better assess for tumor burden and polymer degradation, all the brains were extracted, paraffin-embedded, and assessed histologically (Fig. 6). Interestingly, a significant reduction in gross tumor volume was noted in the ACF 20% groups, compared to the control, blank polymer and TMZ-XRT groups. An even greater reduction in tumor is present in the combination group, and no residual polymeric wafer was present in the cavity. This finding is consistent with

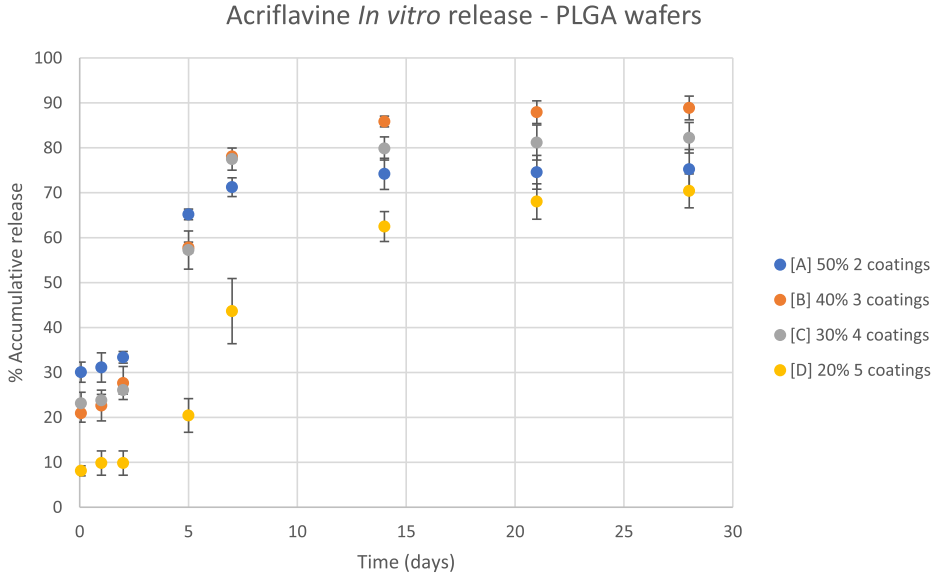

Fig. 4. *In vitro* release of 50%, 40% 30% and 20% Acriflavine HCl in PLGA 75:25 wafers, prepared by 2, 3, 4 and 5 polymer coating, respectively.

Fig. 5. Representative ACF wafer selected for animal studies, before *in vitro* study (left) and after 3 weeks of release (right). Moderate polymer degradation was noted after 3 weeks, nonetheless the remaining mass was still able to hold and release ACF as showed by the polymer color. This constitutes a major improvement over previously employed polymeric platforms.

previous studies in pCPP:SA wafers, but constitutes a new finding is LTS of 9L-implanted F344 rats treated with PLGA wafers.

6. Discussion

6.1. In vitro testing of ACF from biodegradable polymer wafers

Formulation preparation was performed as described previously(16) based upon solubility differences between the drug and the polymer. Briefly, drug particles were dispersed in an organic polymer solution. As solvents evaporated, the polymer formed a coating on the drug particles. Successive coating layers contribute to a homogeneity of the polymer layers around the drug particles. Here, we manipulated the method as the decrease in drug content determined an increase in the number of successive polymer coats.

We confirmed progress of the coating process by increasing the ratio between the C = O peak of the polymer to the C = C peak of the ACF in the FTIR spectra (Fig. 3). Furthermore, the elements on the surface of the produced polymers were detected by EDX. The percentages of Nitrogen and Oxygen detected on the surface of the polymers was used as a surrogate to estimate the extent of O-rich PLGA coverage over the N-rich ACF. Our experiments demonstrated that one coat of PLGA was insufficient to completely cover the ACF, as evidenced by 10.11 % N detected on the surface of the polymers after one round of coating. After a second PLGA coat, %N decreased in favor of %O from the PLGA. This trend was observable as up to five coating layers were introduced (Table 2).

PLGA has been a well-researched polymer for years as a biodegradable and FDA-approved polymer for drug delivery. The knowledge about its properties, behavior and degradation have been thoroughly researched[17]. In this work we used PLGA consisting of a D,L-lactic acid/glycolic acid at ratio of 75/25. The Mw is about 14,000 Da. According to the literature, PLGA, as a polyester, degrades through bulk or heterogeneous erosion in aqueous medium[16,18]. In our *in vitro* study, the ACF wafers, which were compressed and compact at the beginning, increased their dimensions and became swollen and porous (Fig. 4), due to PLGA degradation. The degradation rate in aqueous media is about 5 weeks, confirmed by previous studies[16,18]. According to the *in vitro* results (Fig. 4), the release of ACF has a time scale similar to that of polymer degradation. The aim of this study was to achieve sustained release of ACF from the wafer to prolong the local effects of the drug in an effort to avoid systemic administration and thereby reduce more systemic and non-specific side effects. The formulation [D] containing 20% ACF and 5 polymer coatings released 70% of the loaded drug over 4 weeks. Moreover, there was no burst release at the beginning of the study, indicating successful coating by PLGA. The polymeric envelope better protected ACF from direct contact with water, slowing the release and degradation of this molecule. Formulation [D] was therefore selected for continued *in vivo* studies.

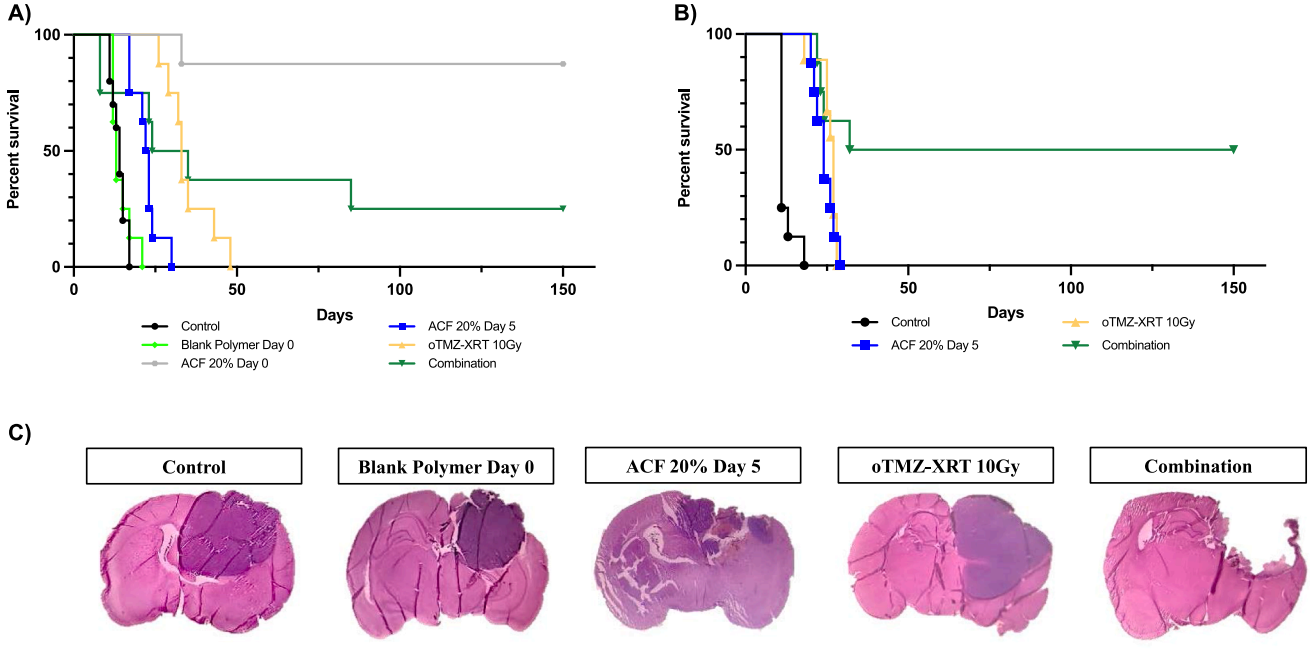

Fig. 6. Kaplan-Meier Curves and Histological Sections. The combination of local ACF and systemic oTMZ-XRT achieved LTS in 9L-implanted rats, when compared to single treatments and combination oTMZ-XRT. **A)** Efficacy study with intracranially implanted 9L tumors on day 0. Groups included untreated (n = 10), empty polymeric wafer (n = 8), 20% ACF-loaded polymer implanted on day 0 (n = 8), 20% ACF polymer implanted on day 5 (n = 8), oTMZ and XRT (n = 8), and 20% ACF polymer, oTMZ and XRT (n = 8). **B)** A second efficacy study for replication. Groups included untreated (n = 8), local 20% ACF on Day 5 (n = 8), oTMZ-XRT (n = 8) and a combination cohort with local ACF, oTMZ and XRT (n = 8). Both studies demonstrated safety of local ACF delivery and significant LTS. **C)** Significant decrease in tumor burden is noted in the ACF 20% in the day 5 group, compared to control and TMZ-XRT groups. Further, no gross residual tumor is visible in the combination group. Polymeric wafer degradation is complete and not visualized in LTS.

6.2. Combination ACF-TMX-XRT therapy prolongs LTS in vivo

HIF-1α, a crucial mediator in the hypoxia cascade, is a key pathway in triggering neovascularization and hypoxia-adaptation in high-grade gliomas, and it is thought to play a role in Cancer-Stem Cell (CSC) chemoresistance and "*peri*-necrotic niche" activation in conditions of low tissue oxygen[19–22]. ACF, an FDA-approved compound with antiseptic properties[23] has recently been repurposed as an HIF-1α inhibitor and anti-cancer therapeutic[24–30]. In particular, previous research shed light on its efficacy against gliomas, when administered intracranially to a 9L tumor model as a monotherapy[10]. This study used p(CPP:SA, 20:80), an FDA-approved method of local drug delivery which is intracranially biocompatible and causes no systemic or local toxicity, to achieve an extraordinary survival benefit in 9L gliosarcoma when intracranially co-implanted with the tumor graft. Currently, p (CPP:SA, 20:80) is clinically utilized for the local delivery of BCNU (Gliadel®), the only interstitial chemotherapy approved for malignant gliomas[4,5].

Nonetheless, the effectiveness and safety of ACF in combination with other commonly used treatment strategies against GBM has not been evaluated *in vitro* and *in vivo*, and its investigation is of vital importance in translating this compound to human use. Furthermore, despite the significant increase in survival of animals treated with locally-administered ACF, questions persist on the ability of current polymeric platforms to administer this therapeutic load safely and gradually over extended periods of time.

In order to address these points, we synthesized a novel polymeric wafer loaded with different percentages of ACF, ranging from 0% to 50% and using 10% incremental increases in weight/weight ratio. Polymeric wafers were implanted intracranially in naïve F344 rats, and their effects compared to those of empty control discs. The highest loading dose tolerated *in vivo* was found to be 20% of the total wafer weight, and this loading was adopted for the *in vivo* efficacy studies. Interestingly, tumor-bearing animals implanted with ACF-loaded PLGA wafers did not show any early deaths immediately post-polymer implantation, a finding in contrast with previous data using pCPP:SA-based polymers, where burst release likely accounted for early animal deaths in treatment groups. Furthermore, 90% of rats treated with 20% ACF wafers at day 0 reached LTS at day 150 post-implantation, confirming the efficacy of this small molecule in targeting tumor cells. These data are directly comparable to those obtained in our previous study, where a few early deaths were noted in the days immediately after wafer implantation. In this case, the only death recorded was a few weeks out from the initial treatment, and likely related to tumor growth, as shown by microscopy.

Nonetheless, with these *in vivo* experiments we aimed to further explore this compound and its potential translatability to humans by evaluating its safety and efficacy in conditions similar to those encountered in patients with GBM. To this extent, a combination of ACF wafer, oTMZ and XRT was compared to the ACF wafer alone and to one of the most common therapeutic protocols currently used in clinic, oTMZ and XRT[3]. Interestingly, while the combination of these three measures did not achieve a superior median survival when compared to TMZ-XRT, a larger fraction of tumor-bearing animals reached LTS at day 150. In the first experiment, about 25% of this group survived until day 150, while ~45% of the second cohort met criteria for LTS.

When compared to the ACF and TMZ-XRT groups these results provide an important proof of the potential translatability of local and systemic combined therapies to larger animal models and eventually to humans. Importantly, and in contrast with previous data, this remarkable LTS was achieved in a 5-day 9L tumor model. The time between initial gliosarcoma implantation and polymer placement allows 9L not only to become more aggressive and resistant to chemotherapeutics – tumor cells have the opportunity to grow and invade the surrounding brain – but the model as a whole more closely resembles the clinical behavior of high-grade gliomas. These results, therefore, bear more clinical relevance, given the closer resemblance of this tumor model to the biology and behavior of human GBM, and their translation to clinical practice. Finally, no local or systemic side effects were recorded during the *in vivo* experiments, providing important evidence that our new PLGA-based polymeric wafer provided a safe platform for local drug delivery in the brain.

7. Conclusions

In conclusion, this study gives evidence regarding a series of issues common to hydrophilic anticancer compounds, such as ACF, and demonstrates the efficacy of local ACF therapy as an adjunct to common anti-GBM regimens. The findings presented here suggest that: 1) a combination of ACF, TMZ, and XRT achieves higher levels of cell necrosis and apoptosis than single treatments; 2) the use of a novel polymeric formulation allows for more gradual and safe release of ACF to the tumor bed, improving local and systemic toxicities; and 3) *in vivo* combination of ACF, TMZ, and XRT significantly prolongs LTS in an aggressive model of rat 9L gliosarcoma, when compared to a common treatment regimen (TMZ-XRT) and local ACF monotherapy only. Future steps will involve the use of larger animal models to validate these findings and optimize drug release to the brain and tumor bed. This will also allow further refinement of *in vivo* polymer kinetics and translation of this technology for human use. Finally, these steps may translate into first-in-human application of this repurposed therapeutic, likely in post-resection recurrent high-grade gliomas.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. However, Dr. H. Brem is a paid consultant to Insightec and chairman of the company's Medical Advisory Board. Insightec is developing focused ultrasound treatments for brain tumors. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict-of-interest policies. Research funding from NIH, Johns Hopkins University, Acuity Bio Corp* and philanthropy. Consultant for AsclepiX Therapeutics, StemGen, InSightec*, Accelerating Combination Therapies*, Catalio Nexus Fund II, LLC*, LikeMinds, Inc.*, Galen Robotics, Inc.* and Nurami Medical (*includes equity or options). B. Tyler has research funding from NIH and is a co-owner for Accelerating Combination Therapies*. Ashvattha Therapeutics Inc. has also licensed one of her patents (*includes equity or options).

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Authors' contributions

RS, AM, NLG, SFT performed experiments, acquired, analyzed, interpreted the data, and wrote the manuscript. RS, AM, SFT, AD, BT and HB designed the experiments, analyzed and interpreted the data, and wrote the manuscript. AD, HB and BT provided cell lines, reagents, technical support, and conceptual advice. All authors approved the final version of the manuscript.

Data availability

All cell lines and materials included in these studies will be freely shared with any interested party. The data that support the findings of this study are available from the authors.

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