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Local delivery of cancer-cell glycolytic inhibitors in high-grade glioma

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Background. 3-bromopyruvate (3-BrPA) and dichloroacetate (DCA) are inhibitors of cancer-cell specific aerobic glycolysis. Their application in glioma is limited by 3-BrPA's inability to cross the blood-brain-barrier and DCA's dose-limiting toxicity. The safety and efficacy of intracranial delivery of these compounds were assessed.

Methods. Cytotoxicity of 3-BrPA and DCA were analyzed in U87, 9L, and F98 glioma cell lines. 3-BrPA and DCA were incorporated into biodegradable pCPP:SA wafers, and the maximally tolerated dose was determined in F344 rats. Efficacies of the intracranial 3-BrPA wafer and DCA wafer were assessed in a rodent allograft model of high-grade glioma, both as a monotherapy and in combination with temozolomide (TMZ) and radiation therapy (XRT).

Results. 3-BrPA and DCA were found to have similar IC_{50} values across the 3 glioma cell lines. 5% 3-BrPA wafer-treated animals had significantly increased survival compared with controls ($P = .0027$). The median survival of rats with the 50% DCA wafer increased significantly compared with both the oral DCA group ($P = .050$) and the controls ($P = .02$). Rats implanted on day 0 with a 5% 3-BrPA wafer in combination with TMZ had significantly increased survival over either therapy alone. No statistical difference in survival was noted when the wafers were added to the combination therapy of TMZ and XRT, but the 5% 3-BrPA wafer given on day 0 in combination with TMZ and XRT resulted in long-term survivorship of 30%.

Conclusion. Intracranial delivery of 3-BrPA and DCA polymer was safe and significantly increased survival in an animal model of glioma, a potential novel therapeutic approach. The combination of intracranial 3-BrPA and TMZ provided a synergistic effect.

Keywords: 3-bromopyruvate, dichloroacetate, glioma, glycolytic inhibitor, pCPP:SA.

Dramatic improvements in cancer survival rates have been achieved over the past 40 years, with a one-year median survival for all cancers in 1971 climbing to a predicted 6-year median survival today.[1] Colon cancer has proven to have the greatest improvement, from a median survival of 7 months in 1971 to a current estimate of 10 years. Encouraging improvements in survival for primary brain malignancies have also been noted, but not to such a profound extent. Despite significant advances in neuroimaging, surgical technique, radiation therapy, and conventional chemotherapy, median survival for WHO grade IV astrocytoma (glioblastoma multiforme [GBM]) remains <15 months.[2] With maximal therapy, consisting of surgical debulking followed by radiation therapy and combination of systemic and local chemotherapy, a median survival of 21 months has been attained at the Johns Hopkins Hospital Brain Tumor Center (Baltimore, Maryland).[3] While improvements have been achieved over the past 2 decades, we must continue the extensive search for novel therapies that target both the aggressive nature and chemotherapeutic resistance of malignant gliomas.

One potential target for glioma therapy is based on the concept of aerobic glycolysis. Highly malignant cells are known to obtain the majority of their energy through the metabolism of glucose directly to lactic acid even in the presence of oxygen, rather than oxidative phosphorylation.[4] GBMs display a strong glycolytic phenotype with an estimated a 300% increase in glycolytic metabolism relative to normal brain.[5,6] This phenotype at first seems counterintuitive because glycolysis generates

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only 2 ATP molecules for every molecule of glucose in contrast to the >30 ATPs produced through oxidative phosphorylation.[7] Theories have been proposed regarding the evolutionary advantage to malignant cells of the additional role of glycolytic enzymes as regulators of transcription and apoptosis.[8] The breakdown of glucose also provides many of the cellular building blocks required for production of the proteins, nucleic acids, and lipids required for replication.[7] In addition, aerobic glycolysis facilitates extracellular acidosis, selecting for cancer cells with more resistant phenotypes that can withstand anaerobic conditions once the oxygen supply has been outgrown.[9] Some glycolytic enzymes have also been noted to play a role in regulating apoptosis. With the upregulation of glycolysis, therefore, malignant cells would be less likely to undergo cell death.

Hexokinase (HK) is the first regulatory enzyme within glycolysis that catalyzes the conversion of glucose to glucose-6-phosphate, capturing glucose molecules within the cell. HK has 4 isoforms: I, II, III, and IV. HK II is found to be upregulated in many tumor cell lines[10] and primarily promotes anabolic functions such as glycogen and lipid synthesis.[11] Wolf et al recently found HK II to be a key mediator of aerobic glycolysis in GBMs, as opposed to predominant HK I expression in normal brain tissue and low-grade gliomas.[12] 3-Brompyruvate (3-BrPA) is a potent ATP inhibitor and direct inhibitor of HK II.[13,14] 3-BrPA has been shown to be well tolerated in vivo at doses required to significantly inhibit tumor ATP production, decrease lactic acid production, and promote apoptosis, with little noted toxicity to normal tissue at therapeutic doses.[15–17] While shown to be effective in glioma cell lines,[16] a limitation of the applicability of 3-BrPA to GBM therapy is its inability to cross the blood-brain barrier.[18]

A second molecule for targeting aerobic glycolysis is dichloroacetate (DCA), which inhibits the mitochondrial enzyme pyruvate dehydrogenase kinase (PDK).[19] PDK is a direct inhibitor of pyruvate dehydrogenase (PDH), which regulates the passage of pyruvate into mitochondria. Through the inhibition of an inhibitor, therefore, DCA promotes mitochondrial oxidation and decreases lactic acid formation.[19] In essence, DCA promotes the aerobic metabolism of glucose through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, in place of the conversion of pyruvate into lactate. Due to the widespread distribution of oral DCA in the central nervous system,[20,21] its efficacy at inhibiting growth in several GBM cell lines has been investigated.[6] DCA led to rapid reversal of mitochondrial hyperpolarization in GBM cells (a marker of an apoptosis-resistant state), promoted p53 activation, and suppressed angiogenesis. In a trial of 3 participants with recurrent GBMs and 2 with primary GBMs, DCA was administered, in addition to standard chemotherapeutic and radiation protocols, and found to possibly be associated with prolonged radiological regression and stabilization. The dose of DCA, however, was limited by reversible peripheral neuropathy. Peripheral nerve toxicity had previously been noted during in vivo and human studies involving disorders of lactic acidosis.[22,23]

Due to the reported inability of 3-BrPA to cross the blood-brain barrier and the dose-limiting toxicity of DCA, we set out to establish whether these molecules could be safely and effectively delivered locally at the tumor site. To maximize clinical relevancy, we selected a proven, FDA-approved method of local drug release using the biodegradable polyanhydride, poly-(1,3 bis[p-carboxyphenoxy] propane-co-sebacic acid) (p[CPP:SA, 20:80]).[24] P(CPP:SA, 20:80) has been shown to be biocompatible in the brain with no evidence of systemic or local toxicity. In multiple glioma studies no difference in survival has been noted between groups receiving empty p(CPP:SA) wafers and untreated animals.[25,26]

We present the tolerability and efficacy of locally delivered 3-BrPA and DCA via p(CPP:SA, 20:80) polymer wafer. We then investigate the combination of 3-BrPA and DCA with temozolomide and radiation therapy.

Materials and Methods

In Vitro Cytotoxicity Analysis

The cytotoxicity of 3-BrPA and DCA were analyzed in the rodent 9L gliosarcoma (Brain Tumor Research Center, University of California at San Francisco) and F98 glioma (R. Barth, Ohio State University) cell lines as well as the human glioblastoma cell line U87 (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum, penicillin/streptomycin (Invitrogen), and L-glutamine (Invitrogen) and kept humidified at 37°C with 5% CO_2.

Cells were plated in 96-well black-bottom plates (BD Falcon) at 2500 cells/well for the 9L and F98 cell lines and 1500 cells/well for the U87 cell line. After 24 hours, 3-BrPA and DCA in dimethyl sulfoxide were added at a concentration range of 0.0001 mM–0.3 mM for 3-BrPA and 0.1 mM–1 M for DCA. Cell counts were measured using the Cyquant cell proliferation assay (Invitrogen) at 450 nm with a microplate reader (Perkin Elmer Victor 3 Wallac) at 48 and 72 hours. Results were repeated no fewer than 3 times. Data were reported as mean inhibitory concentration 50% (IC_{50}) ± 95% confidence interval.

Incorporation of 3-BrPA and DCA into Biodegradable Polymer Wafers

p(CPP:SA, 20:80) (Eisai) was synthesized by melt polycondensation, as previously described.[27] To incorporate 3-BrPA into the polymer matrix, an appropriate amount of 3-BrPA was dissolved with pCPP:SA polymer into a 1:40 solution of methanol in methylene chloride. DCA was similarly incorporated using a 1:13 solution of methanol in methylene chloride. Resulting solutions were then placed in a vacuum desiccator until dried. The polymer mixtures were pressed into 10 mg cylindrical wafers and stored at −20°C.

In Vitro Release of 3-BrPA and DCA from Biodegradable Polymer Wafers

One 5% 3-BrPA wafer and one 50% DCA wafer were placed in separate vials containing 1 mL of phosphate-buffered saline (PBS) in triplicate at 37°C. Equal volumes of PBS were removed and replaced at set time points. PBS samples were frozen at −20C until analyzed. The methods used for measurement of the released 3-BrPA and DCA are detailed in the *Supplementary Material*. Sample preparation involved a single liquid extraction

using acetonitrile. Phenylbutyric acid was used as an internal standard for both 3-BrPA and DCA.

Animals

Female F344 rats, each weighing 160–200 grams, were purchased from Harlan Bioproducts. All rats were housed in standard facilities and provided free access to rodent chow and Baltimore city water. All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

Safety of Intracranial Implanted 3-BrPA and DCA Polymer Wafers

3-BrPA wafers were prepared in concentrations of 1%, 5%, 10%, 25%, or 50% 3-BrPA by weight. DCA wafers were prepared in concentrations of 10%, 25%, or 50% DCA by weight. 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/mLxylazine (Lloyd Laboratories); and 14.25% ethyl alcohol in 0.9% NaCl. The heads were shaved with clippers and prepared with Prepodyne solution (West Penetone). All surgical procedures were performed using standard sterile techniques. After a midline scalp incision, the galea overlying the left cranium was swept laterally. 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 dural incision was made, and the polymer was placed into the brain parenchyma. The animals were evaluated postoperatively every day for at least 120 days. Animals were closely monitored for signs of toxicity, including failure to thrive and neurological deficits. Survival was assessed, and necropsies were performed.

Effect of Intracranial Delivery of 3-BrPA on L-lactate Production

We measured the L-lactate level in brain extracts using the Lactate Assay Kit, EnzyChrom (BioAssay Systems) according to the manufacturer's protocol. The brain lysates were obtained from 18 rats bearing 9L intracranial gliosarcoma tumors. Five days after tumor implantation, the rats were randomized as follows: 9 were intracranially implanted with a 5% Br-PA wafer, and 9 received no treatment (controls). Rats were euthanized on day 6, 24 hours after the polymer implantation, at which point most 3-BrPA would have been released (according to in vitro release kinetics). We defined the tumor as both visible tumor and surrounding peritumoral tissue (<1 mm); the contralateral side consisted of the caudate/putamen area, along with the surrounding white matter. These were dissected and processed separately. The number of cells in each sample was assessed using the Trypan blue exclusion method (ViaCell). The lactate levels were expressed in mM/million cells.

Efficacy of Locally Delivered 3-BrPA and DCA Polymer Wafers

9L gliosarcoma was maintained as a subcutaneous mass, which was passaged every 3–4 weeks in the flanks of F344 rats. For intracranial implantation, the tumor was surgically excised from the carrier animal and sliced into 2 mm^3 allografts. For intracranial implantation, 270 F344 rats (41 rats for Study 1, 48 rats for Study 2, 32 rats for Study 3, and 149 rats for Study 4) were anesthetized and prepped as described above. Under microscope magnification, an opening was made through the dura and cortex, and a small area of cortex was resected. A 2 mm^3 tumor allograft was placed in the resection cavity. In the polymer treatment groups, either one 5% 3-BrPA wafer or one 50% DCA wafer was placed at the time of tumor implantation. The skin was closed with surgical staples. In the oral DCA treatment group, rats received 80 mg/kg/day of DCA delivered via daily oral gavage for days 0–death (dose as previously published).[21,28,29] Rats receiving oral temozolomide (TMZ) were dosed 50 mg/kg via daily gavage for days 5–9.[30] All animals were evaluated daily postoperatively for up to 120 days. Brains were removed and preserved in 10% formalin for analysis.

In Study 1, rats were randomized as follows: group 1 ($n = 9$) received no treatment; group 2 ($n = 8$) received oral DCA; group 3 ($n = 8$) received 5% 3-BrPA wafer; group 4 ($n = 8$) received 50% DCA wafer; and group 5 ($n = 8$) received oral DCA and 5% 3-BrPA wafer. Study 2 assessed the combination of 3-BrPA and DCA polymer, and groups were designated as follows: group 1 ($n = 8$) received no treatment; group 2 ($n = 8$) received oral TMZ; group 3 ($n = 8$) received 5% 3-BrPA wafer; group 4 ($n = 8$) received 50% DCA wafer; group 5 ($n = 8$) received 5% 3-BrPA wafer and 50% DCA wafer; and group 6 ($n = 8$) received 5% 3-BrPA wafer + 50% DCA wafer + oral TMZ. To assess effects of 3-BrPA in combination with TMZ, Study 3 included the following groups: group 1 ($n = 8$) received no treatment; group 2 ($n = 8$) received oral TMZ; group 3 ($n = 8$) received 5% 3-BrPA wafer; and group 4 ($n = 8$) received 5% 3-BrPA wafer + oral TMZ.

Combination 3-BrPA or DCA Polymer Wafers with Systemic Temozolomide and Radiation Therapy

Study 4 investigated the combination of 5% 3-BrPA wafer and 50% DCA wafer with oral TMZ and radiation therapy (XRT) to more closely model clinical therapeutic regimens for high-grade glioma. Rats were implanted with 9L tumor and randomized to one of the following groups: group 1 ($n = 8$) received no treatment; group 2 ($n = 10$) received oral TMZ; group 3 ($n = 8$) received XRT; group 4 ($n = 9$) received IP 3-BrPA 12 mg/kg; group 5 ($n = 9$) received 5% 3-BrPA wafer on day 0; group 6 ($n = 8$) received 5% 3-BrPA wafer on day 5; group 7 ($n = 10$) received oral DCA; group 8 ($n = 10$) received 50% DCA wafer on day 0; group 9 ($n = 8$) received 50% DCA wafer on day 5; group 10 ($n = 9$) received oral TMZ with XRT; group 11 ($n = 10$) received combination IP 3-BrPA + oral TMZ + XRT; group 12 ($n = 10$) received combination 5% 3-BrPA wafer on day 0 + oral TMZ + XRT; group 13 ($n = 10$) received combination 5% 3-BrPA wafer on day 5 + oral TMZ + XRT; group 14 ($n = 10$) received combination oral DCA + TMZ + XRT; group 15 ($n = 10$) received combination 50% DCA wafer on day 0 + oral TMZ + XRT; and group 16 ($n = 10$) received combination 50% DCA wafer on day 5 + oral TMZ + XRT.

XRT was performed using a ^{137}Cs laboratory irradiator (JL Shepard Mark 1 Irradiator, model 68) at a dose of 20 Gy.[30] Animals receiving XRT were anesthetized and placed at a fixed

distance from the radiation source with a collimated beam (1 cm in diameter) centered at the allograft site. The remaining body was shielded with lead.

Statistical Analysis

In vitro cytotoxicity results are reported as the inhibitory concentration 50% (IC_{50}) values for each cell line with the associated coefficient of determination (R^2). In vitro release of drug is reported as the mean and standard deviation plotted against each time point.

L-lactate levels per condition are expressed as mM/million cells. The values are reported as mean ± SEM for 9 different samples per condition, with each tested in duplicate. One-way ANOVA and Tukey' post hoc test were used to analyze the data. *Graphs* were plotted and statistics calculated with GraphPad Prism software, version 6.1. Survival was the primary endpoint in all in vivo efficacy experiments. Kaplan-Meier analysis was used to analyze survival using GraphPad Prism software. Groups were compared using the Mantel-Cox test with 2-tailed *P* value. Differences were considered statistically significant at $P < .05$.

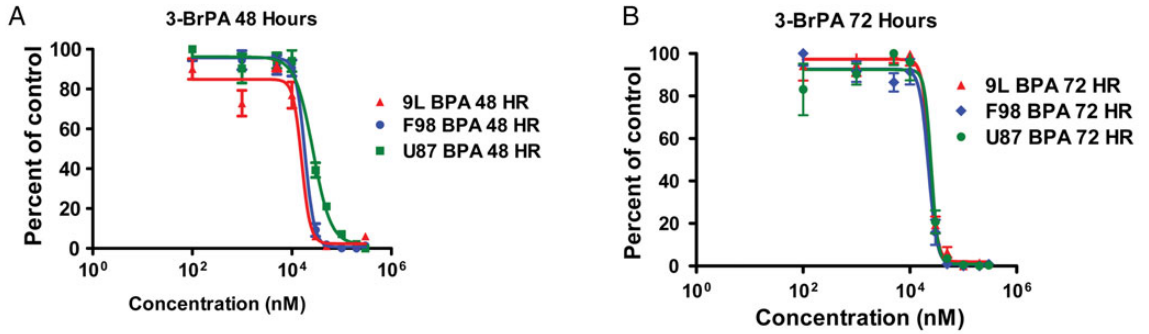
Results

In Vitro Cytotoxicity Analysis

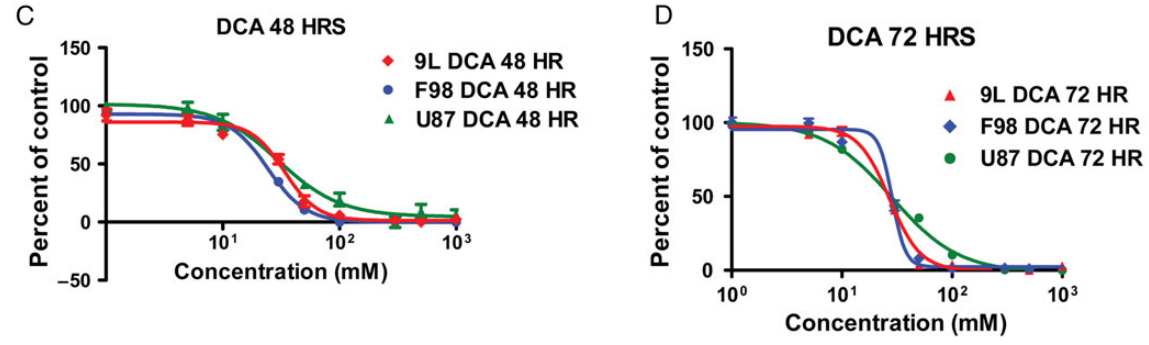
3-BrPA inhibited the growth and proliferation of 2 rodent glioma cell lines and the human glioma cell line (Fig. 1). The IC50 value for 3-BrPA was 15.8–25.5 μM at 48 hours, $R^2 = 0.941–0.971$. Similar IC50 values were found at 72 hours: IC50 = 22.3–25.0, $R^2 = 0.910–0.959$. The IC50 values for DCA at 48 hours were 24.2 mM for F98 and 33.7 mM for 9L with R^2 values of 0.994 and 0.948, respectively. Similar IC50 values were noted at 72 hours.

In Vitro Release of 3-BrPA and DCA from Biodegradable Wafers

In vitro release showed that the majority of 3-BrPA and DCA were released within the first 10 hours, followed by a steady,



	9L 48hrs	F98 48hrs	U87 48hrs		9L 72hrs	F98 72hrs	U87 72hrs
IC 50 (μM)	15.8	18.3	25.5		23.5	22.3	25.0
*95% CI**	11.6 – 21.6	15.2 – 22.1	22.7 – 31.0		14.3 – 38.7	15.5 – 31.9	10.4 – 60.2
R^2	0.941	0.971	0.955		0.959	0.956	0.910



	9L 48hrs	F98 48hrs	U87 48hrs		9L 72hrs	F98 72hrs	U87 72hrs
IC 50 (mM)	33.7	24.2	29.7		28.6	27.0	28.4
*95% CI**	30.1 – 37.7	22.9 – 25.6	23.3 – 37.8		27.4 – 29.8	24.8 – 29.3	25.8 – 31.3
R^2	0.948	0.994	0.923		0.982	0.982	0.989

Fig. 1. Cyquant assay results of 9L rodent gliosarcoma, F98 rodent glioma and U87 human glioma cell lines. (A and B) Results at 48 and 72 hours with increasing concentrations of 3-bromopyruvate (3-BrPA) from 0.0001 mM–0.3 mM. (C and D) Results at 48 and 72 hours with increasing concentrations of dichloroacetate (DCA) from 0.1 mM–1 M. The mean inhibitory concentration 50% (IC 50) for each cell line is reported with the 95% confidence interval (95% CI). The coefficient of determination (R^2) of the linear regression is noted.

slow release of the remainder over the course of the next 3 weeks (*data shown in the Supplementary Section*). This pCPP:SA polymer has previously been published as having a 2-phase degradation process:[31] Water enters the polymer matrix and hydrolyzes the bonds (<10 h), and then the polymer breaks down and dissolves into solution (>3 weeks). In vivo, the polymer components have been shown to take 6–8 weeks to be entirely absorbed.[31]

In Vivo Safety of Intracranial Implanted 3-BrPA and DCA Wafers

Animals implanted with 1% or 5% 3-BrPA wafers displayed no systemic toxicity, however, toxicity was seen at concentrations of 10%, 25%, and 50% 3-BrPA, with median survival <15 days. The maximally tolerated dose for intracranial delivery of DCA was not reached. Wafers containing the maximal loading dose of 50% DCA revealed no neurological or systemic toxicity. Rats treated with 5% 3-BrPA or 50% DCA displayed normal weight gain with 100% survival to 120 days.

In Vivo Effect of Intracranial 3-BrPA Delivery on L-lactate Production

The mechanism of 3-BrPA cytotoxicity is not fully understood, but its ultimate effect, blocking glycolysis, is undisputed. The end product of glycolysis is lactate, and the level of lactate reflects glycolytic activity. We performed an in vivo study to measure the lactate level in brain extracts and, specifically, to compare the lactate level in the tumor side with the corresponding contralateral side (Fig. 2). Eighteen brains obtained from rats bearing 9L gliosarcoma were used, 9 of which received no treatment and 9 that received a 5% 3-BrPa wafer on day 5. The lactate level in the tumor of the control groups was significantly higher than the contralateral side (0.89 ± 0.1 vs 0.60 ± 0.05 mM, $P < .05$). In rats with 5% 3-BrPA, the lactate level in the tumor was significantly lower compared with the contralateral side (0.43 ± 0.02 mM vs 0.60 ± 0.05 mM, $P < .005$) as well as when compared with the tumor location in untreated rats (0.43 ± 0.02 mM vs 0.89 ± 0.1 mM, $P < .005$). These data confirm results that were previously shown only in vitro.[32,33] These data also provide direct evidence of the antiglycolytic action of 3-BrPA, as well as indirect evidence of its cytotoxicity and effective intracranial release in an in vivo glioma model.

In Vivo Efficacy of Locally Delivered 3-BrPA and DCA Wafers

In Study 1, intracranial 5% 3-BrPA and 50% DCA wafers both significantly improved survival in animals with 9L gliosarcoma (Table 1) (5% 3-BrPA median survival, 18 days; $P = .0027$and 50% DCA median survival, 17 days; $P = .02$) relative to the control group (median survival, 13 days) Animals treated with oral DCA (median survival, 11 days) did not demonstrate an improvement compared with the control group. When the 5% 3-BrPA wafer was combined with oral DCA, the combination therapy (median survival, 16 days) was not found to increase survival in comparison with the 5% 3-BrPA wafer (Fig. 3).

Study 2 confirmed that the group receiving the 5% 3-BrPA wafer and the group receiving the 50% DCA wafer had median survivals of 19 days, which significantly prolonged survival compared with the untreated control group (median survival, 14 days; $P < .01$). Combining 5% 3-BrPA and 50% DCA wafers did not provide a survival benefit over either therapy alone. In addition, the efficacy of 5% 3-BrPA and 50% DCA wafer implantation combined with oral TMZ therapy was assessed. This triple combination was well tolerated and resulted in significantly prolonged survival as compared with the controls ($P = .01$). However, it was not found to provide any survival benefit as compared with animals that received oral TMZ only (Fig. 4A).

Study 3 was conducted to assess combined therapy of intracranial 5% 3-BrPA and oral TMZ. Both 5% 3-BrPA wafer and oral TMZ significantly prolonged survival as compared with the untreated controls ($P < .001$). The combination therapy of 5%

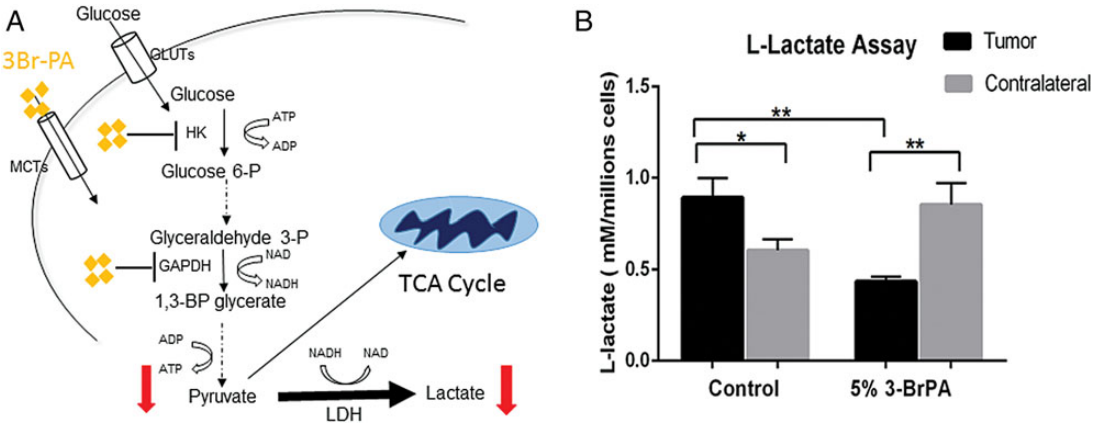


Fig. 2. (A) Schematic representation of the effect of 3-BrPA on the metabolism of glioma cells. 3-BrPA has been shown to inhibit both hexokinase (HK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[32,33] The 3-BrPA inhibits aerobic glycolysis and leads to a decrease of ATP and lactate production. (B) In vivo intracranial measurement of L-lactate. Figure depicts the contralateral and ipsilateral (tumor) areas and their respective lactate levels measured at 565 nm on day 6 and normalized to cell number. Values are shown as mean ± SEM of 9 brains per each condition, each tested in duplicate.

Table 1. Treatment of 9L rat gliosarcoma with 3-bromopyruvate, dichloroacetate, temozolomide, and radiation therapy*

Group	Median Survival, Days (Range)	*P* Value	Long-term Survivors (*n*)
Study 1			
Control ($n = 9$)	13 (10–17)		0
80 mg/kg oral DCA ($n = 8$)	11 (10–15)	NS vs control	0
5% 3-BrPA wafer ($n = 8$)	18 (11–24)	.003 vs control	0
50% DCA wafer ($n = 8$)	17 (11–24)	.02 vs control	0
Oral DCA + 5% 3-BrPA wafer ($n = 8$)	16 (11–24)	NS vs 5% 3-BrPA	0
Study 2			
Control ($n = 8$)	14 (12–19)		0
5% 3-BrPA wafer ($n = 8$)	19 (14–26)	<.01 vs control	0
50% DCA wafer ($n = 8$)	19 (19-LT)	<.001 vs control	1
Oral TMZ ($n = 8$)	19 (16–26)	<.01 vs control	0
5% 3-BrPA wafer + 50% DCA wafer ($n = 8$)	21 (13-LT)	NS vs 5% 3-BrPA wafer; NS vs 50% DCA wafer	2
5% 3-BrPA wafer + 50% DCA wafer + Oral TMZ ($n = 7$)	19 (14–27)	NS vs oral TMZ	0
Study 3			
Control ($n = 8$)	12 (11–17)		0
Oral TMZ ($n = 8$)	25 (20–31)	<.0001 vs control	0
5% 3-BrPA wafer day 0 ($n = 8$)	21 (13-LT)	<.001 vs control	1
5% 3-BrPA wafer day 0 + TMZ ($n = 8$)	119 (32-LT)	<.05 vs 5% 3-BrPA wafer; <0.01 vs oral TMZ	4
Study 4			
Control ($n = 8$)	11 (10–13)		0
Oral TMZ ($n = 10$)	21 (17–27)	<.0001 vs control	0
XRT ($n = 8$)	26 (13–29)	<.001 vs control	0
i.p. 3-BrPA ($n = 9$)	9 (6–11)	<.01 vs control	0
5% 3-BrPA wafer day 0 ($n = 9$)	26 (10-LT)	<.01 vs control	1
5% 3-BrPA wafer day 5 ($n = 8$)	14 (10–17)	<.01 vs control	0
Oral DCA ($n = 10$)	13 (10–16)	<.05 vs control	0
50% DCA wafer day 0 ($n = 10$)	21 (16-LT)	<.0001 vs control <.0001 vs oral DCA	1
50% DCA wafer day 5 ($n = 8$)	10 (10–14)	NS vs control	0
Oral TMZ + XRT ($n = 9$)	34 (16–112)	<.0001 vs control; <.01 vs TMZ; <.05 vs XRT	0
IP 3-BrPA + TMZ + XRT ($n = 10$)	9 (8–41)	NS vs control; NS vs i.p. 3-BrPA	0
5% 3-BrPA wafer day 0 + TMZ + XRT ($n = 10$)	29 (26-LT)	<.05 vs 5% 3-BrPA wafer day 0; NS vs TMZ + XRT	3
5% 3-BrPA wafer day 5 + TMZ + XRT ($n = 10$)	29 (9–93)	<.01 vs 5% 3-BrPA wafer day 5; NS vs TMZ + XRT	0
Oral DCA + TMZ + XRT ($n = 10$)	40 (26-LT)	<.0001 vs Oral DCA; NS vs TMZ + XRT	1
50% DCA wafer day 0 + TMZ + XRT ($n = 10$)	43 (26-LT)	<.05 vs 50% DCA wafer day 0;NS vs TMZ + XRT	2
50% DCA wafer day 5 + TMZ + XRT ($n = 10$)	40 (19-LT)	<.0001 vs 50% DCA wafer day 5; NS vs TMZ + XRT	2

Abbreviations: * 3-BrPA, 3-bromopyruvate; DCA, dichloroacetate; LT, long-term survivor(s); NS, no statistical significance; TMZ, temozolomide; XRT, radiation therapy.

3-BrPA wafer with oral TMZ significantly improved survival over either therapy alone ($P < .05$) (Fig. 4B).

Combination 3-BrPA or DCA Wafers with Systemic Temozolomide and Radiation Therapy

Study 4 was designed to determine additional therapeutic benefit attained from treatment with 3-BrPA or DCA, combined with TMZ and XRT (Fig. 5). While no statistical difference in survival was noted when the wafers were added to the combination therapy of TMZ and XRT, the 5% 3-BrPA wafer given on day 0 in combination with TMZ and XRT resulted in long-term survivorship of 30%.

Discussion

This study presents the successful integration of 2 cancer cell glycolytic inhibitors into biodegradable wafers for local, intracranial chemotherapeutic delivery. These results demonstrate that intracranial 3-BrPA and DCA wafers are well tolerated and significantly increase survival relative to the control group and systemic delivery of these compounds in a rodent model of glioma. In combination with oral TMZ, the intracranial 3-BrPA wafer was found to significantly improve survival over both therapies alone. When both 3-BrPA and DCA wafers were combined with the current clinical regimen for high-grade glioma, oral TMZ and XRT, both were well tolerated. The addition of either glycolytic inhibitor with TMZ and XRT treatment did not provide any statistical survival advantage as compared with

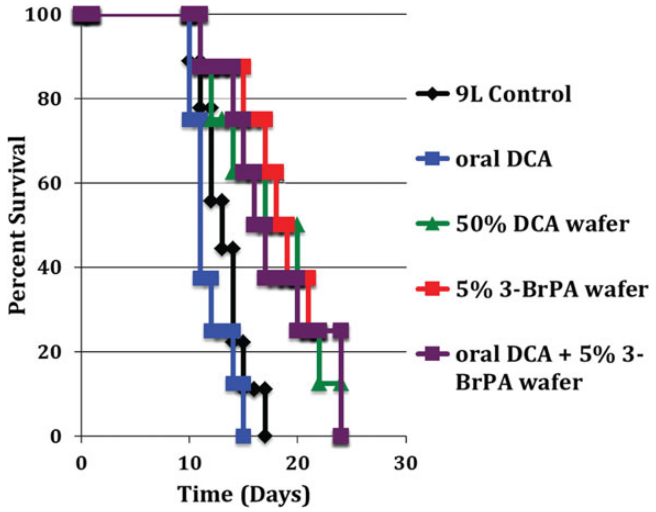


Fig. 3. Survival Study 1: Efficacy of intracranial, controlled release of dichloroacetate (DCA) and 3-bromopyruvate (3-BrPA) in the 9L rodent gliosarcoma model. Kaplan-Meier plots of F344 rats that were implanted with 9L and either given no treatment (9L Control, $n = 9$) or were randomized as follows: 80 mg/kg/day of DCA via gavage on days 0-death (oral DCA, $n = 8$), 50% DCA wafer on day 0 ($n = 8$, 5% 3-BrPA wafer on day 0 ($n = 8$), or combined 80 mg/kg/day of DCA with 5% 3-BrPA wafer ($n = 8$).

those receiving TMZ and XRT, although the triple combination therapy was associated with a few long-term survivors. The treatment strategy assessed through this series of experiments is based on the following factors: (i) the route of administration (local release of 3-BrPA and DCA) and (ii) the potential for additional therapeutic benefit when glycolytic inhibitors are given in combination with systemic TMZ and XRT.

Effect of 3-BrPA on Cancer Cell Metabolism and the Necessity for Local Delivery

Several oncoproteins have been recognized to induce glycolytic enzymes, which in turn have been shown to play a regulatory role in cancer cell apoptosis.[8] One oncogene in particular, AKT, has been found to promote both aerobic glycolysis and inhibit apoptosis by both transcriptional and nontranscriptional mechanisms.[8,34] AKT inhibits apoptosis in part by negatively regulating the enzyme, glycogen synthase kinase 3 beta (GSK3beta). Inhibition of GSK3beta promotes the binding of HK II to the outer mitochondrial membrane.[35] HK II, in contrast to other forms of hexokinase, contains a N-terminal hydrophobic domain that allows binding to the outer mitochondrial membrane protein, voltage-dependent anion channel (VDAC). The binding of HK II to VDAC yields a 5-fold increase in its affinity to ATP and provides feedback inhibition from G-6-P. VDAC has also been found to be a key protein in mitochondria-mediated apoptosis with HKII acting in an apoptosis-suppressive capacity.[36,37] 3-BrPA has been found to cause dissociation of HK II from VDAC and significantly inhibit the phosphorylated, active form of AKT.[38] Therefore, 3-BrPA is able to decrease cancer cell aerobic glycolysis and promote apoptosis.[39] 3-BrPA has also been noted to interact with other intracellular proteins as an alkylating agent.[40]

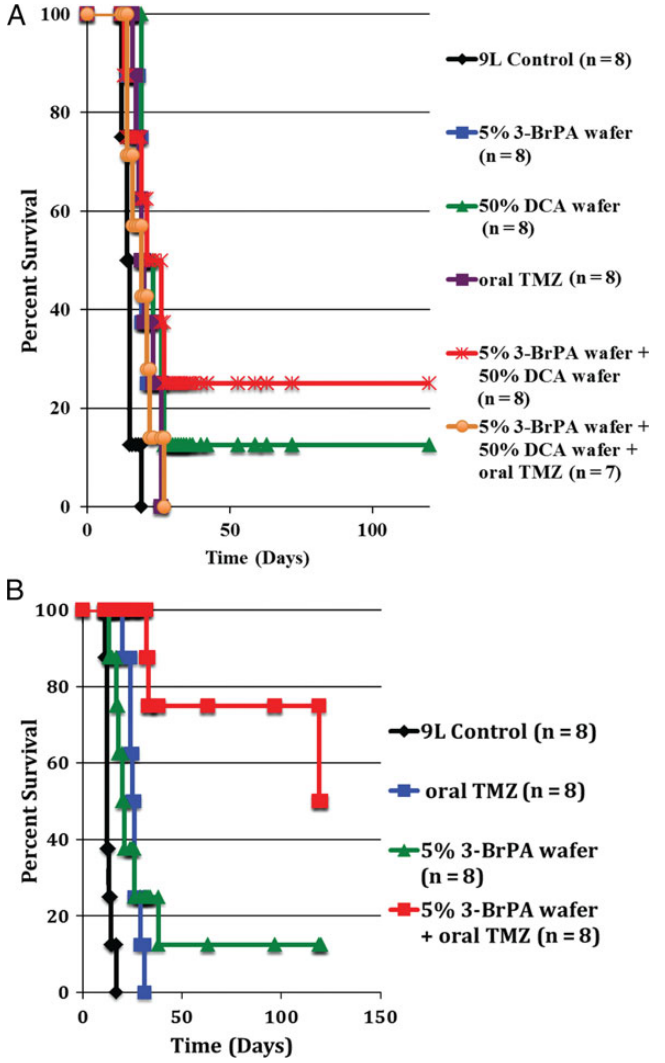


Fig. 4. Survival Studies 2 and 3: Efficacy of combined local delivery of both 3-bromopyruvate (3-BrPA) and dichloroacetate (DCA) via controlled-release polymer wafer and efficacy of combined delivery of 3-BrPA wafer with temozolomide (TMZ). (A) Kaplan-Meier plots of F344 rats that were implanted with 9L gliosarcoma either given no treatment (9L Control, $n = 8$) or randomized as follows: 5% 3-BrPA wafer ($n = 8$), 50% DCA wafer ($n = 8$), 50 mg/kg/day of temozolomide (TMZ) via gavage on days 5–9 (oral TMZ, $n = 8$), both 5% 3-BrPA wafer and 50% DCA wafer simultaneously ($n = 8$), or combined TMZ with both 5% 3-BrPA wafer and 50% DCA wafer ($n = 8$). (B) Kaplan-Meier plot of Study 3: F344 rats implanted with 9L gliosarcoma either given no treatment (9L control, $n = 8$) or randomized as follows: 50 mg/kg/day of temozolomide (TMZ) via gavage on days 5–9 (oral TMZ, $n = 8$), 5% 3-BrPA wafer given on day 0 (5% 3-BrPA, $n = 8$), or combined oral TMZ with 5% 3-BrPA wafer ($n = 8$).

Effective 3-BrPA therapy for glioma requires determination of sufficient drug delivery to the brain for maximal cytotoxic effect against the tumor cells. Vali et al assessed the biodistribution using radio-labeled (^{14}C)3-BrPA and found a consistently low uptake in the highly metabolic tissue of the brain, suggesting poor penetration of the blood-brain barrier.[18] Dose escalation studies of 3-BrPA in rodents have revealed systemic toxicity

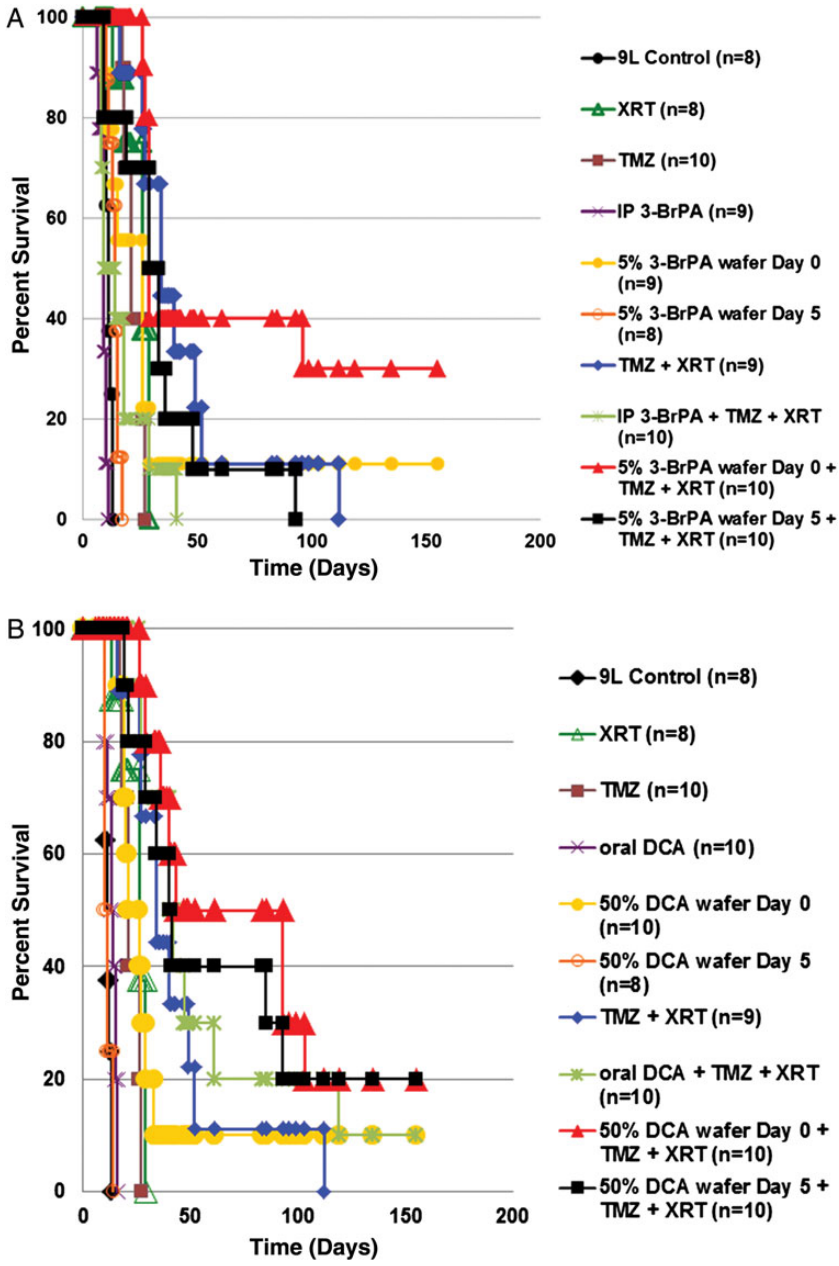


Fig. 5. Survival Study 4: Efficacy of 3-bromopyruvate (3-BrPA) or dichloroacetate (DCA) via polymer wafer with systemic temozolomide (TMZ) and radiation therapy (XRT). Results from the single survival study are separated into A and B plots with the same no-treatment control and oral TMZ and XRT treatment groups for purposes of simpler comparison among relevant groups. (A) Kaplan-Meier plots of F344 rats implanted with 9L gliosarcoma either given no treatment (9L Control, $n = 8$) or randomized as follows: 20 Gy of XRT on day 5 (XRT, $n = 8$), 50 mg/kg/day of TMZ via gavage on days 5–9 (TMZ, $n = 10$), 12 mg/kg/day of 3-BrPA via intraperitoneal injection on days 0–4 (IP 3-BrPA, $n = 9$), 5% 3-BrPA wafer day 0 ($n = 9$), 5% 3-BrPA wafer day 5 ($n = 8$), TMZ with XRT on day 5 ($n = 9$), 3-BrPA IP with TMZ and XRT ($n = 10$), 5% 3-BrPA wafer day 0 with TMZ and XRT ($n = 10$), or 5% 3-BrPA wafer day 5 with TMZ and XRT ($n = 10$). (B) Kaplan-Meier plots from the same experiment presenting rats given no treatment (9L Control, $n = 8$) or randomized as follows: 20 Gy of XRT day 5 (XRT, $n = 8$), TMZ 50 mg/kg/day, days 5–9 (TMZ, $n = 10$), DCA 80 mg/kg/day via gavage on days 0–death (oral DCA, $n = 10$), 50% DCA wafer day 0 ($n = 10$), 50% DCA wafer day 5 ($n = 8$), TMZ with XRT on day 5 ($n = 9$), oral DCA with TMZ and XRT ($n = 10$), 50% DCA wafer day 0 with TMZ and XRT ($n = 10$), or 50% DCA wafer day 5 with TMZ and XRT ($n = 10$).

at doses >20 mg/kg with death at doses >30 mg/kg.[41] In our study, 3-BrPA at a systemic dose of 12 mg/kg/day, a dose found to be efficacious for in vivo tumor models outside of the CNS,[15,41] was not found to be effective in the 9L intracranial gliosarcoma model. The potential solution is local drug delivery. Local administration of 3-BrPA was initially described as trans-arterial delivery in a rabbit model of liver cancer.[18] Intracranial local delivery of 3-BrPA has not yet been described. Our study

demonstrates that intracranial controlled-release of 3-BrPA at a concentration required to achieve a survival benefit is safe. The 5% 3-BrPA wafer consistently provided a >30% survival benefit in the rodent 9L gliosarcoma model. As previously noted, the rapid release of 3-BrPA within the first 24 hours is consistent with the release of other compounds from p(CPP:SA 20:80) such as 1,3-bis(2-chloroethyl)-1-nitrosourea.[31]

Role of DCA in Promoting Oxidative Phosphorylation and Benefit of Local Delivery

Aerobic glycolysis relies on increased lactic acid production in place of the mitochondrial-based oxidative phosphorylation. The gate-keeping enzyme PDH regulars whether pyruvate, the product of glycolysis, is broken down to lactic acid or enters the mitochondria to undergo the TCA cycle and electron transport chain.[21] PDH functions to decarboxylate pyruvate to acetyl-coenzyme A in order for it to enter the TCA cycle. The flow of electrons down the electron transport chain is associated with production of reactive oxygen species (ROS). PDH is inactivated by its phosphorylation via PDK. DCA, an inhibitor of PDK, has been found to inhibit lactic acid production and promote oxidative phosphorylation.[19] Oxidative phosphorylation has been associated with increased production of ROS and efflux of proapoptotic mediators from mitochondria with induction of mitochondria-dependent apoptosis in cancer cells but not in normal cells.[21]

DCA at a dose of 50 mg/kg/day was found to possibly prolong radiographic regression and stabilization in human patients with GBM. Unfortunately, the dose was associated with reversible peripheral neuropathy.[6] DCA administered orally at a dose of 80 mg/kg/day was found to decrease the tumor size in a rodent model of subcutaneous adenocarcinoma, although animal survival was not analyzed.[21] We did not find that 80 mg/kg/day of oral DCA provided a survival benefit in the intracranial 9L rodent gliosarcoma model, which is perhaps evidence for a higher systemic dose requirement for glioma therapy. Higher systemic doses, however, may entail even greater toxicity. DCA in a 3-month chronic administration study was found to have a lethal dose of 2000 mg/kg in rats, while a lethal dose of 75 mg/kg was noted in dogs.[42] The success seen with the local delivery of DCA via a 50% DCA wafer suggests that local delivery of DCA may be required to achieve an efficacious dose at the tumor site without the toxicity associated with systemic DCA delivery.

Combination of Glycolytic Inhibitors with Temozolomide and Radiation Therapy

The combination of 3-BrPA wafer and DCA, both in oral and polymer wafer form, with TMZ and XRT may warrant further investigation because 3-BrPA wafer in combination with TMZ was found to provide a synergistic effect when compared with either TMZ or 5% 3-BrPA wafer alone. Glioma stem cells are thought to be a major contributor to the resistance of gliomas to standard treatments.[2] The chemoresistant state may be due to the upregulation of multidrug resistant genes, which leads to reduced intracellular drug accumulation through increased expression of membrane efflux transporters, as well as the inhibition of apoptosis.[43,44] Nakano et al recently elucidated that glycolysis may be related to cancer cell chemoresistance.[45] Since the function of membrane efflux transporters is dependent on ATP and ATP production in tumor cells is largely based on aerobic glycolysis, Nakano et al hypothesized that inhibition of glycolysis will preferentially decrease the efflux of anticancer agents, allowing for maximal therapeutic effect. They found that 3-BrPA restored the cytotoxic effect of daunorubicin and doxorubicin in resistant tumor cell lines expressing membrane efflux transporters. Indirect evidence from numerous studies has shown that enzymes targeted by 3-BrPA are implicated in TMZ chemoresistance, which suggests that TMZ could be potentially more effective when combined with 3-BrPA.[46,47] Our study shows that 3-BrPA enhances TMZ efficacy in vivo. This synergistic and chemosensitizing effect is either due to HKII inhibition,[54] which has been shown to sensitize glioma cells to TMZ, or to the downregulation of p-Akt, which plays a key role in the overexpression of a GLUT 3 transporter[46] and Mcl-1,[47,48] an anti-apoptotic Bcl-2 family related to TMZ resistance.

Radioresistance is thought to be a common property of cancer stem cells, possibly due to their ability to more efficiently repair DNA relative to noncancer stem cells.[49] It has been noted that cancer stem cells also contain lower levels of ROS due to enhanced expression of free radical scavenger systems. ROS are the predominant mediators of cell death induced by ionizing radiation; therefore, enhanced scavenging of free radicals may result in increased radioresistance.[50] El Sayed et al recently showed that 3-BrPA increases H_2O_2 production, and thus increases ROS.[16] Similarly, DCA has been shown to increase whole cell H_2O_2 within GBM cell lines.[6]

Conclusion

Both 3-BrPA and DCA were found to be cytotoxic in vitro in a human glioma cell line as well as rodent glioma and gliosarcoma cell lines. These glycolytic inhibitors were successfully integrated into pCPP:SA polymer, which allowed for their intracranial delivery. In vivo experiments showed a significant increase in survival with 5% 3-BrPA and 50% DCA wafers in the intracranial 9L rodent gliosarcoma model. 5% 3-BrPA wafer in combination with TMZ significantly increased survival as compared with either therapy alone and in combination with TMZ and XRT, while no statistical difference was found, and an increase in the number of long-term survivors was noted. Oral DCA and 50% DCA wafer, given in combination with TMZ and XRT, also yielded a few long-term survivors. Further studies should be conducted to investigate the local delivery of 3-BrPA and DCA in additional cell lines and to assess potential mechanisms for increased efficacy of combined local delivery of 3-BrPA and DCA with current standard clinical regimens.

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Conflict of interest statement. The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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