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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Epirubicin exhibits potent anti-tumor activity in an animal model of malignant glioma when administered via controlled-release polymers

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Abstract Epirubicin (EPI) has strong cytotoxic activity that makes it a potential candidate for the treatment of malignant gliomas. To minimize toxicity and increase CNS penetration, EPI was incorporated into biodegradable polymers, and its in vitro and in vivo properties were studied. 9L, F98, C6, U251, and EMT-6 cell lines were treated with EPI in vitro and cell viability was measured. Toxicity of EPI/polycarboxyphenoxypropane-sebacic-acid (pCPP:SA) polymers was tested in vivo using F344 rats intracranially implanted with EPI polymers (2–50% by weight). The efficacy of 50% EPI:pCPP:SA polymers was determined in F344 rats intracranially challenged with 9L and treated either simultaneously or 5 days after tumor implantation. The efficacy of 50% EPI:pCCP:SA polymers administered on Day 5 in combination with oral TMZ was determined in rats intracranially challenged with 9L gliosarcoma. EPI was cytotoxic in all cell lines used in vitro. Intracranial implantation of the EPI polymers in rats generated neither local nor systemic toxicity. Animals receiving intracranial EPI on Day 5 had 50% long-term survivors (LTS), which was superior to local EPI delivered on Day 0 (LTS = 12.5%). Animals receiving intracranial EPI in combination with oral TMZ had 75% LTS whereas no other group had LTS. In those EPI treated animals that died before the controls there was evidence of intracranial hemorrhage. Systemic epirubicin resulted in high toxicity levels and early deaths in all the experiments. EPI polymers, alone or in combination with oral TMZ, is an effective therapeutic modality against experimental 9L gliosarcoma.

Keywords Epirubicin · Biodegradable polymer · Brain tumor · Interstitial chemotherapy

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Introduction

Malignant gliomas, the most common primary brain tumors, are usually fatal malignancies associated with rapid growth and aggressive clinical behavior [1]. With the new treatments the median survival has increased from 9 to 20 months [2–6]. In addition to surgical resection and radiotherapy, numerous chemotherapeutic agents have been utilized in an attempt to treat this disease [7], but drug limitations including poor central nervous system penetration and dose limiting toxicities, have restricted their use [8].

One way of combating gliomas, while avoiding these limitations, is through local delivery of chemotherapeutic agents into the central nervous system (CNS), utilizing drugs impregnated into a controlled release polymer-matrix. With this process drugs approved by the FDA for treatment of various forms of cancer are impregnated into a polymer matrix and implanted into the CNS.

Epirubicin is an anthracycline commercially available as Ellence®. It is indicated for use in breast cancer patients with axillary node tumor involvement [9]. Epirubicin intercalates into the DNA causing the helix to become distorted and thereby inhibits DNA replication, RNA

synthesis, and cell division [10]. Anthracyclines have also been found to be part of the most effective regimens for the treatment of advanced gastric cancer [11]. However, the use of epirubicin is limited by its myelotoxic effects [9] and associated cardiotoxicity [12]. It has been documented that 10 to 26% of patients who receive cumulative doses of epirubicin above the recommended dose of 1000 mg/m^2 develop congestive heart failure, and over half of the patients who receive this cumulative dose of epirubicin will experience measurable functional impairment months to years following treatment [9]. The potential of epirubicin to treat brain tumors when delivered systemically is also limited by its inability to cross the blood-brain barrier [11, 13]. We reasoned, however, that direct intracranial delivery of epirubicin via a polymer would lead to improved efficacy in an animal model of malignant glioma.

It has previously been demonstrated that carmustine (BCNU) prolongs survival in patients with malignant gliomas when it is delivered via biodegradable polymer wafers at the time of tumor resection [5, 14–18]. We hypothesized that local delivery of epirubicin via a biodegradable polymer wafer will also allow this powerful agent to be used for the treatment of brain tumors.

In this study, we investigated epirubicin's cytotoxicity against experimental glioma cell lines, we incorporated the drug into biodegradable polymers, assessed the pharmacokinetics of this formulation in vitro, and established its CNS toxicity in vivo. In order to mirror current clinical practice of using a combination of therapies to treat malignant gliomas, we used epirubicin polymers in combination with oral temozolomide (TMZ). TMZ is an alkylating agent that has shown a statistically significant prolongation of survival in patients with malignant gliomas when given orally [19].

Materials and methods

Tumor cell lines

Rat 9L gliosarcoma cell line was obtained from Dr. M. Barker at the University of California at San Francisco Brain Tumor Research Center (San Francisco, CA). The F98 glioma cell line was obtained from Dr. R. Barth (Ohio State University, Columbus, OH). The C6 glioma was obtained from the American Type Culture Collection (Manassas, VA). The U251 human glioma was obtained from Duke University (Durham, NC). The EMT-6 breast carcinoma cell line was obtained from the DCT Tumor Repository (NCI-Frederick Cancer Research and Development Center, Frederick, MD). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum media, and replaced when an appropriate density was reached according to the suggestions of the provider.

Cytotoxicity experiments

In order to quantify the efficacy of epirubicin, in vitro cytoproliferation experiments were performed. 9L, F98, C6, U251, and EMT-6 cell lines were used in this study. Using one plate per cell line, 3500 cells/well were plated onto 96-well plates on Day 0. Cells were incubated for 24 h at 37°C. On Day 1, epirubicin at concentrations of 2.5, 1.25, 0.625, 0.3125, and 0.15625 μg/ml were added to ten wells each, while one row of ten wells was treated with an isovolumetric amount of DMEM media to act as controls. Following epirubicin treatment, cells were then placed in a 37°C incubator for a period of 48 h. Cell viability was then quantified using the MTT assay.

MTT cell viability assay

The MTT assay, a measure of mitochondrial integrity that is often lost during apoptosis, was performed to assess cell proliferation. This method provides a quantitative measurement of the number of cells with metabolically active mitochondria and is based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay). After exposure to epirubicin, supernatant from each of the wells was aspirated and replaced with 0.5 mg/ml MTT solution. Cell plates were then incubated at 37°C for 4 h. MTT solution was then aspirated and replaced with reagent grade dimethyl sulfoxide. Cell plates were read at 570 nm with an ELISA plate reader (Beckman Coulter, model AD 340, Fullerton CA). This absorbance is linearly proportional to the number of live cells with active mitochondria [20].

Polymer preparation

EPI was incorporated into a polyanhydride CPP:SA (PCPP:SA) polymer at concentrations of 25, 40, and 50% by methods described previously [17]. Briefly, PCPP:SA and epirubicin were combined and dissolved in methylene chloride at room temperature. This solution was dried in a vacuum desiccator for 2–3 h and stored at −20°C. The polymers were molded into disks measuring 3 × 3 × 1 mm and weighing approximately 10 mg. Blank polymers were made in an analogous manner. Polymer-drug formulations with optimal release profiles were selected for further testing.

Release kinetics

In vitro release kinetics were performed to determine both the amount and duration of drug release. The epirubicin polymers were placed into a 1-ml phosphate-buffered saline (PBS) solution at 37°C and samples collected at 1, 3, 6, 9, 24,

and 48 h. Drug released was analyzed by UV spectroscopy. Epirubicin release is expressed as the percentage of loaded concentration ± standard error of the mean (SEM).

Animals and care

F344 female rats, weighing 150–200 g purchased from Harlan Bioproducts (Indianapolis, IN), were used. They were housed in standard facilities and given free access to food and water. All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

Animal toxicity study

Epirubicin was incorporated into biodegradable polymer at 2, 10, 25, 50% w/w. Sixteen animals were divided into groups of 4. Each group underwent an intracranial implantation of a single polymer concentration. Rats were anesthetized with an intraperitoneal injection of 2–4 ml/kg of a stock solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and ethanol (14.25%) in a sterile 0.9% NaCl solution. The heads were shaved and disinfected with 70% ethanol and povidone-iodine solution. After a midline scalp incision, the galea overlying the left cranium was swept laterally. With the aid of an operating microscope, a 3 mm burr hole was made over the left parietal bone, with its center 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. An incision was made in the dura cortex and 10 mg of the polymer was placed subdurally. The scalp incision was then closed with surgical staples. Animals were observed daily for any signs of deterioration, neurotoxicity, or movement disorders. Animals were evaluated pre-operatively, and daily for 40 days to determine signs of local and systemic toxicity. Animals were weighed every 5–7 days. On Day 40 rats were euthanized and their organs were harvested for analysis and fixed in 10% paraformaldehyde.

Intracranial glioma model

The 9L gliosarcoma was maintained in the flanks of Fisher 344 (Harlan Sprague Dawley, Indianapolis, IN) rats. For intracranial implantation, the 9L gliosarcoma tumor was surgically excised from the carrier animal, cut into 1-mm^3 pieces, and placed in sterile 0.9% NaCl on ice. For intracranial implantation of the 9L glioma 167 female Fischer 344 rats, weighing 150–200 g, were anesthetized with an intraperitoneal injection of 3–5 ml/kg of a stock solution containing ketamine hydrochloride 25 mg/ml (Ketlar; Parke-Davis Corporation Morris Plains, NJ), xylazine 2.5 mg/ml (Rompun; Mobay Corp., Shawnee, Kansas), and 14.25% ethyl alcohol in 0.9% NaCl. All surgical procedures were carried out using sterile surgical technique. The head was prepared with alcohol and prepodyne solution, and a midline scalp incision was made, exposing the sagittal and coronal sutures. A small burr hole was made with an electric drill and 2 mm round cutting burr, centered 3 mm lateral to the sagittal suture, avoiding the sagittal sinus, and 5 mm posterior to the coronal suture. The dura was incised and forceps were used to lift off remaining bone and dura. With gentle suction, a small area of cortex and white matter was resected. Once hemostasis was achieved, a single tumor piece (1 mm^3) was placed in the depths of the cortical resection. The skin was then closed with surgical staples.

In vivo efficacy studies

Comparison of local to systemic administration of EPI

To determine the effectiveness of locally delivered epirubicin, the tumor-bearing rats were randomized into three groups for treatment. Eight animals received 50% EPI polymers on postoperative Day 5, five animals received intraperitoneal injection daily for 5 days (Days 5–9) with 1 mg epirubicin hydrochloride solution and six animals received no treatment. Animals were observed for systemic and neurologic toxicity and survival was recorded. Any animals appearing moribund were sacrificed and date of death recorded. At Day 90, the experiment was terminated and all surviving rats were euthanized. Histopathological studies of all animals' brains harvested at the time of death or euthanasia were examined to confirm the presence or absence of tumor.

Efficacy of locally delivered EPI

To determine the effectiveness of locally delivered EPI the tumor-bearing rats were randomized into groups of eight for treatment with blank polymer, 50% EPI polymer at the time of the tumor implantation, 50% EPI polymer 5 days postoperatively, 3.8% BCNU polymer 5 days postoperatively as a positive control for the experimental brain tumor model. Animals were observed for systemic and neurologic toxicity and survival was recorded. Any animals appearing moribund were sacrificed and date of death recorded. At Day 120, all surviving rats were deemed long-term survivors. However the study was allowed to continue to 186 days because the treatment animals looked healthy. Histopathological studies of all animals' brains harvested at time of death or euthanasia were examined to confirm the presence or absence of tumor.

Efficacy of combined treatment with locally delivered EPI and oral TMZ

To determine the efficacy of the combination of locally delivered EPI with oral TMZ, the tumor-bearing rats were randomized into groups of 8 for treatment on postoperative Day 5 with either blank polymer, blank polymer and oral TMZ (50 mg/kg/animal given from Day 5–Day 9), 50% EPI polymer, 50% EPI polymer and oral TMZ, systemic EPI (1 mg/kg/animal given intraperitoneally from Day 5–Day 9), systemic EPI and oral TMZ. Animals were observed for systemic and neurologic toxicity and survival was recorded. Any animals appearing moribund were sacrificed and date of death recorded. At Day 90, all surviving rats were deemed long-term survivors and were euthanized. Histopathological studies of all animals' brains harvested at time of death or euthanasia were examined to confirm the presence or absence of tumor.

Statistical analysis

Statistical analysis was performed using Excel. Cytotoxicity, determined in vitro by the MTT assay, was analyzed using the Student's t test; values were expressed as mean ± standard error of the mean (SEM). Kaplan–Meier curves for the efficacy studies were analyzed based on survival algorithms and significance was determined by the long rank and Kruskal–Wallis tests. A probability value of <0.05 was considered significant for all tests.

Results

In vitro efficacy

For 9L, there was a 68.57 ± 4.28% (Mean ± SEM) maximum growth inhibition at a concentration of 2.5 μg/ml (Fig. 1). For F98, the maximum inhibition was 84.34 ± 3.08% at a concentration of 2.5 μg/ml (Fig. 2); for U251, maximum inhibition was 49.87 ± 3.78% at a concentration of 0.625 μg/ml (Fig. 3); for C6, maximum inhibition was 66.68 ± 3.24% at a concentration of 1.25 μg/ml (Fig. 4); and for EMT-6, maximum inhibition was 92.05 ± 1.97% at a concentration of 2.5 μg/ml (Fig. 5). These values were all statistically significant. Cell

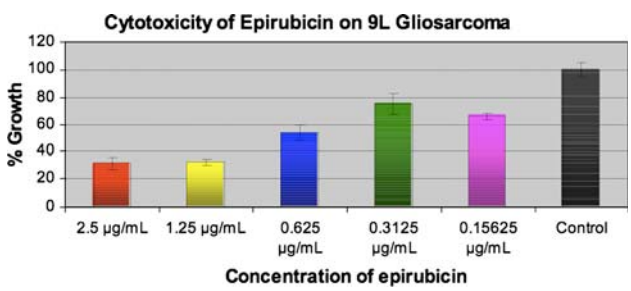

Fig. 1 Cytotoxicity of epirubicin on 9L cells in vitro. Significant growth inhibition of 9L was achieved at all concentrations tested

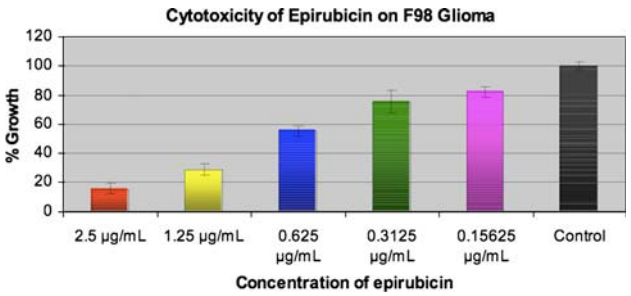

Fig. 2 Cytotoxicity of epirubicin on F98 cells in vitro. Significant growth inhibition of F98 was achieved at all concentrations tested

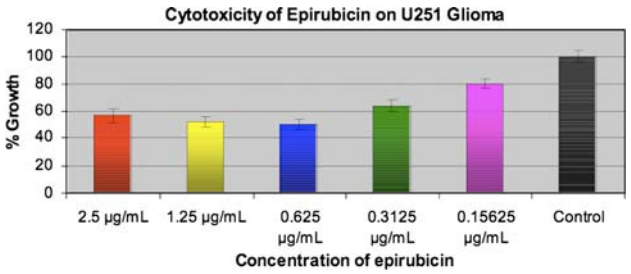

Fig. 3 Cytotoxicity of epirubicin on U251 cells in vitro. Significant growth inhibition of U251 was achieved at all concentrations tested

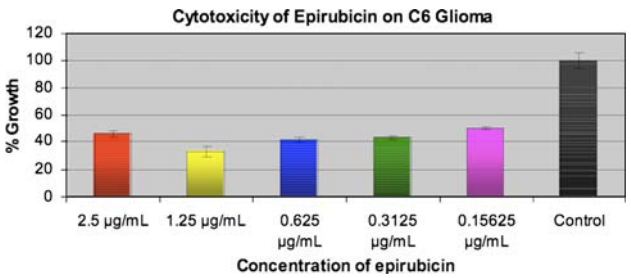

Fig. 4 Cytotoxicity of epirubicin on C6 cells in vitro. Significant growth inhibition of C6 was achieved at all concentrations tested

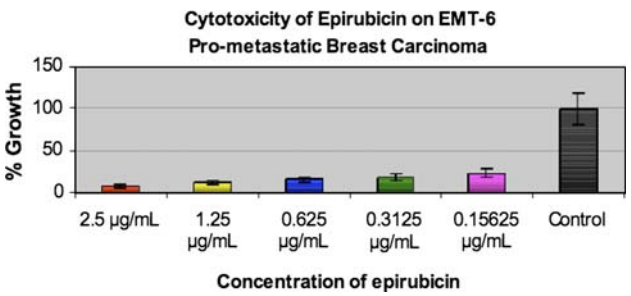

Fig. 5 Cytotoxicity of epirubicin on EMT-6 cells in vitro. Significant growth inhibition of EMT-6 was achieved at all concentrations tested

Table 1 Results of the cytotoxicity study of epirubicin (EPI) on glioma and cancer cell lines

Cell line	Most effective concentration (ug/ml)	% of growth inhibition	% SEM (standard error of the mean) (%)	*P* value
9L	2.5	68.57	±4.28	<0.001
F98	2.5	84.34	±3.08	<0.001
C6	1.25	66.68	±3.24	<0.001
U251	0.625	49.87	±3.78	<0.001
EMT-6	2.5	92.05	±1.97	<0.001

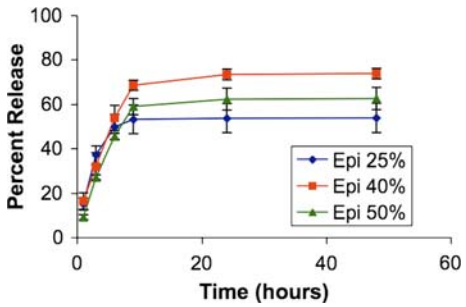

Fig. 6 Pharmacokinetics. The release rate of epirubicin from pCPP:SA 25, 40, 50% (w:w) measured in vitro. Sustained release of epirubicin was observed with all polymer formulations

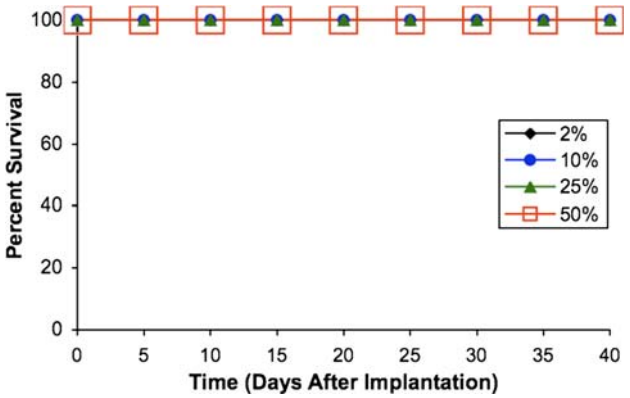

Fig. 7 Kaplan–Meier survival curve. Healthy animals were implanted with different formulations of epirubicin polymers. Animals implanted with 2, 10, 25, 50% (w:w) showed neither systemic nor local toxicity up to the day of euthanasia

growth was inhibited for all concentrations of epirubicin in all other cell lines, and these results were also statistically significant ($P < 0.001$) (Table 1).

Release kinetics

Epirubicin was incorporated into the biodegradable polymer pCPP:SA at various loading percentages: 25, 40 and 50% (w:w). Release kinetics of the polymers all showed an initial burst effect within the first 10 h followed by a small release of drug throughout the rest of the first day. Within 48 h 50% epirubicin:pCPP:SA released 63 ± 9%, 40% epirubicin:pCPP:SA released 74 ± 4%, and 25% epirubicin:pCPP:SA released 59 ± 12% (Fig. 6).

In vivo toxicity study

F344 rats were implanted intracranially with varying loading doses of epirubicin in polymer: 2, 10, 25, and 50%. Each group had four animals. All animals appeared normal throughout the toxicity study and showed no changes in weight (Fig. 7).

In vivo efficacy studies

Comparison of local to systemic administration of EPI

The animals that received treatment on Day 5, either 50% epirubicin polymer or systemic epirubicin (1 mg by intraperitoneal injections, Days 5–9) had a statistically significant increase in survival as compared to control animals ($P = 0.0012$, $P = 0.0095$ respectively), while they had longer median survival and more LTS than any other treatment group. The group receiving 50% EPI polymer had significantly increased survival in comparison to the animals treated with systemic EPI ($P = 0.0415$). Whereas all animals receiving systemic EPI were dead on Day 31 (median survival 31 days), 50% of the animals receiving the EPI polymer were alive on Day 90 (median survival not reached) (Table 2, Fig. 8). Histological analysis of the brains of the animals receiving systemic EPI did not reveal an intracranial cause of death. The mortality associated with systemic EPI appears to be through nonspecific tissue

Table 2 Efficacy of 50% epirubicin (EPI) polymer vs. systemic epirubicin against 9L gliosarcoma

Group	Median survival (Days)	Long term survivors (%)	*P*-value
Control, $n = 6$	13 (11–17)	0	
50% EPI polymer, $n = 8$	Not reached (13–90)	50	0.0012 vs. controls
			0.04 vs. 1 mg EPI IP
1 mg EPI intraperitoneal (IP), $n = 5$	31 (17–31)	0	0.0095 vs. controls
			0.04 vs. 1 mg EPI polymer

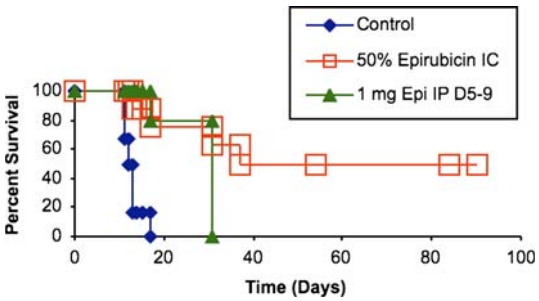

Fig. 8 Kaplan–Meier survival curve. The efficacy of local epirubicin (EPI) was tested in the 9L gliosarcoma using Fisher F344 rats. The animals were divided into three groups and received local EPI, systemic EPI, or no treatment

toxicity rather than as a tumor-specific treatment. Histopathological review of the LTS revealed no evidence of tumor burden upon completion of the study.

Efficacy of locally delivered EPI

The intracranial administration of EPI on Day 5 increased median survival and produced more long-term survivors (LTS) than the groups receiving either 50% EPI polymer on Day 0 or 3.8% BCNU polymer on Day 5 (Fig. 9). Control animals had a median survival of 13 days. The groups receiving treatment on Day 5, both 50% epirubicin (median survival not reached; $P = 0.0013$) and BCNU (median survival = 77; $P = 0.0001$) had a statistically increased survival as compared to control animals. The Day 5 BCNU treatment group yielded 37.5% LTS while the Day 5 EPI group had 50% LTS. There was no significant difference between the treated groups receiving Day 5 BCNU or Day 5 epirubicin ($P = 0.52$). However, both median survival and long term survival were superior in the Day 5 EPI group. The administration of EPI on Day 5

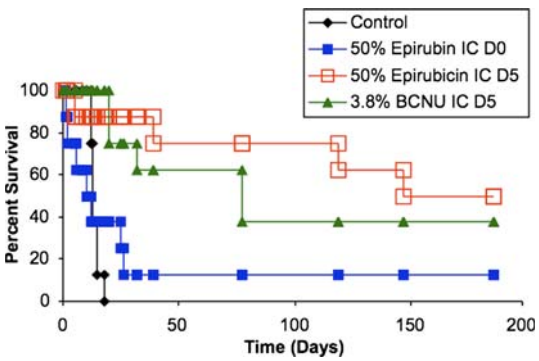

Fig. 9 Kaplan–Meier survival curve. The efficacy of local epirubicin (EPI) given on Day 0 or Day 5 was tested in the 9L gliosarcoma using Fisher F344 rats. The animals were divided into four groups and received local EPI on Day 0, local EPI on Day 5, local BCNU or no treatment

yielded significantly better results than the implantation of the polymer on Day 0 (median survival = 12 days, LTS = 12.5%, $P = 0.0223$). Significant toxicity was observed in the latter group, with four of the eight animals dying before the controls (Table 3, Fig. 9). Histopathological review of the LTS revealed no evidence of tumor burden upon completion of the study.

Efficacy of combined treatment with locally delivered EPI and oral TMZ

Intracranial delivery of 50% EPI polymers and oral TMZ increased median survival and produced more LTS when compared to the animals receiving blank polymer (median survival = 19 days, LTS = 0%, $P < 0.0001$), local EPI alone (median survival = 15 days, LTS = 0%, $P = 0.0012$), oral TMZ and blank polymer (median survival = 30 days, LTS = 0%, $P < 0.0001$), intraperitoneal EPI (median survival = 13 days, LTS = 0%, $P < 0.0001$), intraperitoneal EPI and oral TMZ (median survival = 12 days, LTS = 0%, $P < 0.0001$) (Table 4 and Fig. 10). Consequently the animals treated with the combination of intracranial EPI and TMZ had the longest prolongation of survival with median survival not reached and 75% LTS. However, the animals that received local EPI on Day 5 and died before the controls had intracranial hemorrhage at autopsy. Histopathological review of the LTS revealed no evidence of tumor burden upon completion of the study.

Discussion

Epirubicin (EPI) has been shown to inhibit tumor cell growth in numerous tumor cell lines and is currently used to treat a host of solid and hematologic malignancies including breast [9, 10, 21] and gastric carcinoma [11]. Previous cytotoxicity studies have documented the efficacy of EPI in vitro in C6 glioma cell lines [22, 23]. EPI is a second-generation anthracycline and exerts its cytotoxic action mainly through inhibition of DNA and RNA synthesis after drug intercalation in the DNA [24]. Its tumoricidal activity has also been attributed to inhibition of topoisomerase II [25], metabolic activation to toxic reactive semiquinone free radical intermediates [26] or even membrane transduction of growth inhibition messages [27]. Doxorubicin, which is the major representative of the first-generation of anthracyclines, has been shown to be effective in the treatment of experimental gliomas, when delivered locally [28] or through targeted therapy [29]. The increased effectiveness of EPI in comparison to this first generation of agents makes it an even more attractive means of combating glioblastoma multiforme (GBM). However, its clinical use in the treatment of brain malignancies is limited by its

Table 3 Efficacy of 50% epirubicin (EPI) polymer vs. 3.8% BCNU polymer against 9L gliosarcoma

Group	Median survival (Days)	Long term survivors (%)	*P*-value
Control, $n = 8$	13 (12–18)	0	
50% EPI polymer (Day 0), $n = 8$	12 (1–186)	12.5	0.74 vs. controls
			0.02 vs. 50% EPI (5)
			0.03 vs. 3.8% BCNU (5)
50% EPI polymer (Day 5), $n = 8$	Not reached (5–186)	50	0.0013 vs. controls
			0.02 vs. 50% EPI (0)
			0.52 vs. 3.8% BCNU (5)
3.8% BCNU polymer (Day 5), $n = 8$	77 (20–186)	37.5	<0.0001 vs. controls
			0.02 vs. 50% EPI (0)
			0.52 vs. 50% EPI (5)

Table 4 Efficacy of 50% epirubicin (EPI) polymer in combination with oral temozolomide (TMZ) against 9L gliosarcoma

Group	Median survival (Days)	Long term survivors (%)	*P*-value
Control, $n = 8$	19 (12–21)	0	
50% EPI polymer (Day 5), $n = 8$	15 (8–64)	0	0.84 vs. controls
			0.66 vs. Blank poly + TMZ
			0.0012 vs. EPI poly + TMZ
			0.059 vs. EPI intraperitoneal (IP)
			0.032 vs. EPI IP +TMZ
Blank polymer (Day 5) + oral TMZ, $n = 8$	30 (27–39)	0	<0.0001 vs. controls
			0.66 vs. EPI poly
			<0.0001 vs. EPI poly + TMZ
			0.0061 vs. EPI IP
			0.0014 vs. EPI IP +TMZ
50% EPI polymer (Day 5) + oral TMZ, $n = 8$	Not reached (54–90)	75	<0.0001 vs. controls
			0.0012 vs. EPI poly
			<0.0001 vs. Blank poly + TMZ
			<0.0001 vs. EPI IP
			<0.0001 vs. EPI IP +TMZ
1 mg EPI intraperitoneal (IP), $n = 8$	13 (11–14)	0	0.0061 vs. controls
			0.059 vs. EPI poly
			<0.0001 vs. Blank poly + TMZ
			<0.0001 vs. EPI poly + TMZ
			0.24 vs. EPI IP +TMZ
1 mg EPI IP + oral TMZ $n = 8$	12 (11–13)	0	0.0014 vs. controls
			0.032 vs. EPI poly
			<0.0001 vs. Blank poly + TMZ
			<0.0001 vs. EPI poly + TMZ
			0.24 vs. EPI IP

inability to cross the blood-brain barrier [13] and its dose-dependent systemic toxicity [9, 12]. The use of biodegradable polymers loaded with EPI, in a similar manner as the FDA-approved carmustine wafers (Gliadel®), could theoretically overcome these obstacles [30, 31]. In our current study we hypothesized that EPI is a potent inhibitor of rodent glioma in vitro and in vivo administered in the form of a biodegradable polymer.

Our in vitro results confirmed that EPI has significant cytotoxic activity against rodent and human glioma cell

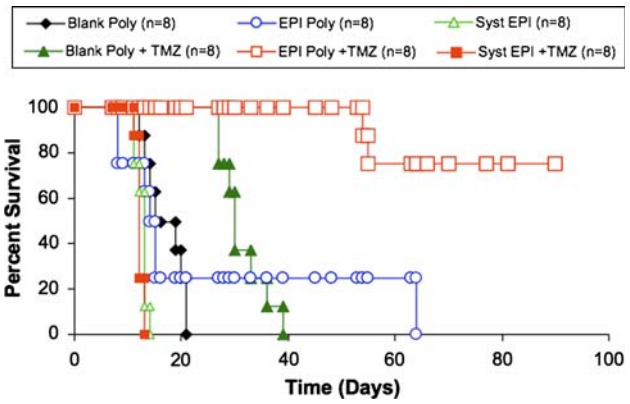

Fig. 10 Kaplan–Meier survival curve. The efficacy of combination of local epirubicin (EPI) and oral TMZ was tested in the 9L gliosarcoma using Fisher F344 rats. The animals were divided into six groups and received blank polymer, blank polymer and oral TMZ, EPI polymer, EPI polymer and oral TMZ, systemic EPI, systemic EPI and TMZ

lines as well as a breast carcinoma cell line frequently used in rodent models of metastatic brain tumors. We found that it inhibited the growth of all cell lines when delivered at 0.15–2.5 μg/ml. The effect was dose dependent and was consistent among all cell lines tested (Table 1). Moreover the LD_{50} was achieved with lower doses than those required for maximal growth inhibition. Subsequently, we made an implantable disk with EPI in concentrations ranging from 25 to 50% and the biodegradable polymer CPP:SA and measured its release profile. Release kinetics of all the polymers showed an initial burst effect within the first 10 h followed by a sustained release of drug throughout the rest of the first day that maximized within 48 h (Fig. 6).

Our in vivo toxicity studies indicated that polymers loaded with 2–50% EPI were well tolerated by Fisher 344 rats, without any signs of local, systemic toxicity or weight changes (Fig. 7). We have expanded these results by comparing the efficacy and toxicity of local versus systemic EPI in rats intracranially challenged with 9L gliosarcoma. As expected, based on the available clinical data, the use of systemic EPI resulted in increased toxicity and early death of the animals. Both median survival and long-term survival associated with local treatment were superior ($P = 0.04$) to the systemic use of EPI. Fifty percent of the locally treated animals were still alive upon completion of the study whereas there were no survivors in the systemically treated group (Table 2, Fig. 8).

In an effort to elucidate the best timing for local treatment we compared the efficacy of 50% EPI polymers implanted either on Day 0 or on Day 5. The former group demonstrated significant toxicity with 50% of the animals dying before the controls (Table 3, Fig. 9). Consequently, the administration of the polymer on Day 5 (median survival not reached, LTS = 50%) proved significantly better in comparison to Day 0 (median survival = 12 days, LTS = 12.5%, $P = 0.0223$). This indicates that in a clinical scenario it might be best if local EPI is administered after surgery to avoid possible toxic effects. The latter, could be the result of the dissemination of EPI in the blood stream due to micro-bleedings caused by the suction of brain tissue during tumor implantation. Alternatively its toxic effects can be attributed to the interaction of EPI with the acutely inflamed tissue during surgery. In the same experiment we included for comparison a group of animals receiving 3.8% carmustine polymers, the only FDA-approved locally delivered chemotherapeutic agent for GBM. Although there was no statistically significant difference ($P = 0.52$) in the effectiveness of BCNU and EPI (Day 5), both median survival and long-term survival associated with the latter were superior. Specifically, the animals of the EPI group did not reach median survival and had 50% LTS, whereas the BCNU group had a median survival of 77 days and 37.5% LTS. Epirubicin's similarity with BCNU in effectiveness is promising for its clinical applicability.

These encouraging results mandated the investigation of the effectiveness of the combination of local EPI administered on Day 5 and oral TMZ. TMZ has been shown to prolong survival when taken orally in patients with malignant gliomas [19, 32]. By combining TMZ, an alkylating agent, with EPI, an anthracycline, we attempted to follow the current trend in chemotherapy to block multiple cellular pathways in order to inflict maximal tumor damage [33].

The combination of the two chemotherapeutic agents prolonged the survival of tumor-bearing animals more than EPI polymer alone ($P = 0.0012$) or blank polymer with oral TMZ ($P < 0.0001$). The group receiving this treatment was the only one not to reach median survival with 75% LTS. We concurrently confirmed our previous results that the systemic administration of EPI in cytotoxic concentrations is highly toxic. All the tumor-bearing animals that received systemic EPI as part of their therapeutic scheme sustained major toxicities with many animals dying earlier than the controls (Table 4, Fig. 10).

Our results need to be examined in consideration of the limitations of the 9L gliosarcoma model. This very frequently used rodent glioma model is characterized by the implantation of a rodent tumor to rats and does not mirror the inherent growth and invasion of human glioma. Promising survival data in these models are the basis for further studies in more complex animal tumor models (or even human glioma models) with higher order organisms such as monkeys. These results would most closely parallel human disease. This would be the necessary next step in studying the efficacy of EPI polymers.

Despite these promising results in overall survival, there was a subgroup of animals receiving local EPI on Day 5 that died earlier than the controls. All these rats had evidence of intracranial hemorrhage at autopsy. This possible complication, which was not observed in the animal toxicity study, may act as a limiting factor in the clinical use of local EPI and requires further investigation in more complex animal models or with a different slower releasing polymeric configuration. However, even if intracranial hemorrhage is consistently established as a possible complication of local EPI use, there are patient groups that can benefit from its use. As an example, patients with recurrent GBM, low Karnofsky performance score (<70), and normal coagulation profiles could be considered as candidates for local EPI use. This recommendation could be justified if the survival benefit of local EPI alone or in combination with local TMZ, which we observe in the animal studies, could be replicated in clinical trials.

Conclusion

In these studies we have shown the effective cytotoxicity of EPI against experimental glioma cell lines and we have designed a successful means of delivering EPI locally, which minimizes systemic side effects and delivers EPI in a controlled, sustained fashion at therapeutic concentrations. We have shown that EPI, when delivered in this manner, significantly prolonged survival of rodents bearing malignant tumors. The combination of EPI with oral TMZ improves survival more than either of these treatments alone. While these initial findings point to the clinical application of EPI in the treatment of malignant gliomas, further work must first be done to investigate CNS toxicity associated with the local delivery of EPI in more complex animal models.

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