Local delivery of mitoxantrone for the treatment of malignant brain tumors in rats

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Object. Mitoxantrone is a drug with potent in vitro activity against malignant brain tumor cell lines; however, its effectiveness as a systemic agent has been hampered by poor central nervous system penetration and dose-limiting myelosuppression. To avoid these problems, we incorporated mitoxantrone into biodegradable polymeric wafers to be used for intracranial implantation, a strategy that has been shown to be safe and successful in the treatment of malignant gliomas. The authors investigated the release kinetics, toxicity, distribution, and efficacy of mitoxantrone delivered from intracranially implanted biodegradable wafers in the treatment of 9L gliosarcoma in Fischer 344 rats.

Methods. Mitoxantrone released from the biodegradable wafer matrix reached therapeutic drug concentrations in the brain for at least 35 days. Only animals with implanted wafers of the highest drug loading dose (20% mitoxantrone by weight) showed signs of significant toxicity. In three separate efficacy experiments, animals treated with mitoxantrone-loaded biodegradable wafers had significantly improved survival compared with control animals. The combined median survival for each treatment group was the following: 0% mitoxantrone wafers, 19 days; 1%, 30 days, $p < 0.0001$; 5%, 34 days, $p < 0.0001$; and 10%, 50 days, $p < 0.0001$.

Conclusions. These findings establish that mitoxantrone delivered from intracranially implanted biodegradable wafers is effective in the treatment of malignant gliomas in rodents and should be considered for future clinical application in humans.

KEY WORDS • mitoxantrone • brain neoplasm • glioma • implantable wafer • rat

MITOXANTRONE is a dihydroxyanthracenedione derivative that is used in the treatment of advanced breast cancer, non-Hodgkin lymphoma, acute nonlymphoblastic leukemia, and chronic myelogenous leukemia in blast crisis,[10] and has been approved for the treatment of hepatic and ovarian cancer.[9] It has also been identified as one of the most potent drugs against malignant glioma cell lines in vitro.[15,20]

Despite its clinical effectiveness in the treatment of various malignancies and its in vitro activity against gliomas, data from clinical trials based on the systemic administration of mitoxantrone for treatment of malignant brain tumors have failed to demonstrate efficacy.[18] Systemic treatments have been hindered by the poor penetration of mitoxantrone into the CNS and dose-limiting myelosuppression with resulting leukopenia. Autopsy findings in patients treated with systemic mitoxantrone revealed that CNS tissues contained the lowest concentrations of the drug, approximately 500-fold less than that of thyroid and liver tissues.[17] Furthermore, tumors contained lower concentrations of mitoxantrone than surrounding normal tissues, and brain tumors contained the lowest drug concentrations of all tumors.

Consequently, we reasoned that a local intracerebral delivery method for mitoxantrone capable of directly bypassing the blood–brain barrier would be able to achieve therapeutic CNS drug concentrations while minimizing systemic exposure. The use of a drug-loaded biodegradable polymer system to deliver treatment locally to brain tumors is a technique that has recently been added to the neurosurgical arsenal against malignant brain tumors.[5,7]

To evaluate the hypothesis that mitoxantrone delivered locally from a biodegradable polymer system could be an effective therapy for malignant gliomas, we investigated the following factors: 1) the efficacy of mitoxantrone against experimental brain tumor cell lines in vitro; 2) the drug release profile of implantable wafers composed of mitoxantrone and the biodegradable polymer pCPP:SA; 3) the toxicity of this system following implantation into the rat brain; 4) the distribution of mitoxantrone in the brain following implantation of mitoxantrone-loaded wafers; and 5) the ability of mitoxantrone delivered from these wafers to extend survival in an in vivo intracerebral malignant glioma model.

Materials and Methods

Tumor Cell Lines

All tumor cell lines used in this study—9L, F98, U87, U251, and

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U373—were cultured in appropriate media, as suggested by their providers.

In Vitro Activity of Mitoxantrone

Inhibition of tumor proliferation was tested with rodent (F98 and 9L) and human glioblastoma cell lines (U87, U251, and U373). Cells were plated in at least triplicate with increasing concentrations of mitoxantrone ranging from 5×10^{-6} to 5 ng/mg in appropriate media. The cells were counted after a 48-hour exposure by using a cell counter and compared with control cells receiving no mitoxantrone.

Wafer Preparation

The matrix pCPP:SA had a 20:80 molar ratio. Mitoxantrone dihydrochloride was combined with pCPP:SA (20:80), according to the mix-melt method[8] to create a thin film containing 0, 1, 5, 10, and 20% mitoxantrone by weight. Cylindrical wafers weighing 10 mg each (3 mm in diameter, 1 mm thick) were then manufactured using a steel molding press.

Drug Release Kinetics

The pCPP:SA wafers loaded with varying concentrations of mitoxantrone were placed in 1.5-ml vials containing 1 ml of 0.1 M phosphate buffered saline, pH 7.4. The vials were capped to prevent evaporation and placed in an incubator at 37°C. The saline solution was periodically removed and replaced to approximate infinite sink conditions. The amount of mitoxantrone released was determined by measuring absorbance at 682 nm on an ultraviolet/visible spectrophotometer[14] and by calculating the drug concentration based on a standard curve.

Animal Care

Eighty-six Fischer 344 rats, weighing between 150 and 200 g, were housed in standard facilities and given free access to water and rodent chow. All rats were treated in accordance with the policies and principles of laboratory animal care of the Johns Hopkins University School of Medicine Animal Care and Use Committee.

Intracranial Tumor Implantation

Rats were anesthetized with an intraperitoneal injection of 2 to 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 a 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 2 to 3 mm posterior to the coronal suture and 3 to 4 mm lateral to the sagittal suture. Great care was taken to avoid injury to the dura mater. The rats were then placed in a stereotactic frame, and 10,000 9L glioma cells were injected over 3 minutes through a 26-gauge needle inserted into the center of the burr hole at a depth of 3 mm. The needle was then removed and the site was irrigated with normal saline. After ensuring hemostasis, the wound was closed with surgical staples.

Wafer Implantation

In animals not receiving tumor cells, following burr hole placement, the dura mater and underlying brain parenchyma were opened using a No. 11 surgical blade. Then, with the aid of an operating microscope, the wafer was placed into the brain parenchyma at a depth of approximately 1 mm below the dura. After ensuring hemostasis, the skin was closed with surgical staples. In tumor-bearing animals, surgical wounds were reopened and wafers were implanted 5 days after injection of tumor cells.

In Vivo Mitoxantrone Wafer Toxicity

To determine the maximally tolerated mitoxantrone loading dose, 20 rats, evenly divided into five groups, underwent intracerebral implantation of wafers containing 0, 1, 5, 10, or 20% mitoxantrone pCPP:SA wafers. Animals were closely monitored for signs of toxicity, including failure to thrive and neurological deficits. Survival was assessed and autopsies were performed whenever possible.

In Vivo Distribution of Mitoxantrone in the Rodent Brain

Twelve rats underwent implantation of 10% mitoxantrone pCPP:SA wafers into the left parietal lobe and were observed daily for signs of neurotoxicity. Three animals were killed on each of Days 3, 7, and 18 following wafer implantation by using an intracardiac injection of pentobarbital (following induction of anesthesia, as described earlier). One animal died on Day 30, thus only two animals were killed on Day 35. Each brain was harvested, wafer remnants were removed, and the brains were snap frozen in heptanes over dry ice.

After collection of all the specimens, the rat brains were thawed and divided in the coronal plane into 2-mm-thick sections centered around the wafer implantation site. Each of these sections was then divided in the midsagittal plane. The sections were individually weighed, minced, and then transferred to separate containers.

Extraction of mitoxantrone from the tissue samples was performed using modified versions of the techniques described by Stewart, et al.[17] Briefly, the minced tissue samples were extracted using an H_2O/acetone (10:1) solution during 96 hours at 37°C. This aqueous solution was acidified with 1 M HCl, washed twice with chloroform, and extracted twice with an equivalent volume of chloroform and 28 to 30% ammonium hydroxide (10:1). The organic layers were combined and evaporated overnight. The remaining residue was taken up with 0.1 M HCl and filtered. Mitoxantrone content was determined with the aid of an ultraviolet/visible spectrophotometer at 682 nm.[14] Extraction efficiency was determined by proceeding through the earlier steps with a known initial quantity of mitoxantrone dihydrochloride. All mitoxantrone concentrations were calculated based on the initial tissue sample mass with corrections for extraction efficiency.

In Vivo Intracranial Efficacy

In three separate experiments, a total of 86 rats were innoculated with 9L gliosarcoma cells. Five days later, wafers containing mitoxantrone (0, 1, 5, or 10%) were implanted directly into the site of tumor cell injection. Animals were observed daily for signs of toxicity. Animals with anorexia or hemiplegia were killed. The end point of these experiments was survival.

Statistical Methods

For in vitro studies, data were analyzed using the two-tailed Student t-test. For animal experiments, survival data were analyzed with the log-rank (Mantel–Cox) test in a Kaplan–Meier nonparametric analysis performed using statistical software.

Sources of Supplies and Equipment

The 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 U87 and U373 tumor lines were obtained from the American Type Culture Collection (Manassas, VA). The U251 glioma was obtained from Duke University (Durham, NC). The cells were counted using a cell counter from Beckman Coulter (Fullerton, CA). The matrix pCPP:SA was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD). The mitoxantrone dihydrochloride was purchased from Sigma-Aldrich Corp. (St. Louis, MO). The ultraviolet/visible spectrophotometer (Genesys 5) used to determine the amount of mitoxantrone released was acquired from Spectronic (Rochester, NY).

The Fischer 344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). The surgical staples used to close the wounds in the rats were purchased from Clay Adams (Parsippany, NJ). Statistical analysis was performed using Statview software (version 4.5) from Abacus Concepts, Inc. (Berkeley, CA).
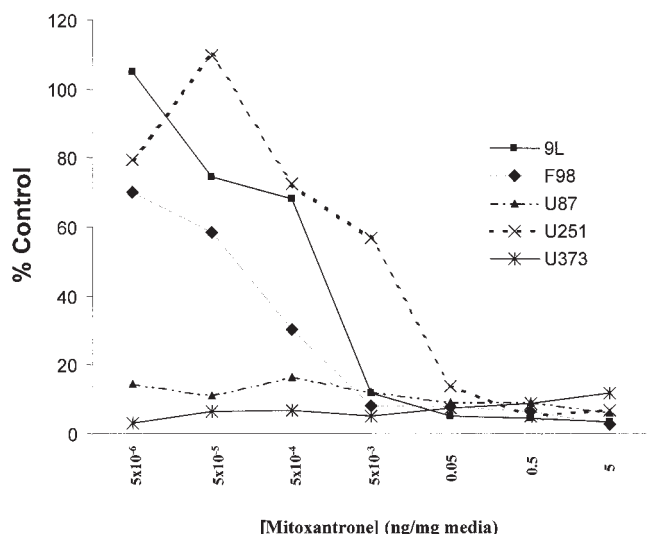
FIG. 1. Graph demonstrating in vitro growth inhibition of rodent (9L and F98) and human glioma (U87, U251, and U373) cell lines induced by incubation with mitoxantrone for 48 hours. Each data point represents the mean value of at least three trials.

Results

In Vitro Efficacy of Mitoxantrone

Mitoxantrone was a potent inhibitor of both human and rodent glioma cell growth. At a concentration of 0.05 ng/mg, all cell lines, except U251, were inhibited by more than 90% (Fig. 1). The U251 cell line was less sensitive, but at 0.5 ng/mg its growth was less than 10% that in controls.

Polymer Formulation and Drug Release Kinetics

With the mix-melt method, the maximum loading dose of mitoxantrone was approximately 20%. Release into buffer for wafers loaded with 1, 5, 10, or 20% mitoxantrone by weight showed a dose-dependent increase in the rate of drug release. Due to the solubility of mitoxantrone in aqueous solutions, drug release was rapid (Table 1). In all cases, however, sustained mitoxantrone release was observed during a period longer than 48 hours.

In Vivo Mitoxantrone Wafer Toxicity

The implantation of 20% mitoxantrone wafers into the brains of four healthy rats resulted in two deaths. Hence, this loading dose was discarded from further study. One animal implanted with 5% mitoxantrone died on Day 19 after the sudden onset of seizures despite an otherwise healthy condition. None of the animals implanted with 1 or 10% mitoxantrone wafers showed any signs of toxicity, although one animal receiving the 10% wafer in the study of drug distribution (see later discussion) died 30 days after implantation.

In Vivo Distribution of Mitoxantrone in the Rodent Brain

The extraction efficiency for mitoxantrone was 70%. The concentrations of mitoxantrone in brain parenchyma following implantation of 10% mitoxantrone wafers were greatest in the ipsilateral hemisphere within 3 mm of the implantation site (Day 3 500–850 ng/mg of brain tissue;

TABLE 1

*In vitro release of various concentrations of mitoxantrone-loaded pCPP:SA wafers**

Time (hrs)	Cumulative % Release of Mitoxantrone		
	1%	5%	10%
2	4.6	7.55	52.6
6	6.7	10.4	68.9
12	7.7	11.4	73.9
24	12.2	11.9	76.6
48	13.0	40.0	77.2
120	13.7	40.8	78.0

* Data represent the mean percentage of release based on total drug loading in three separate trials.

Day 7 550–700 ng/mg; Day 18 125–270 ng/mg; and Day 35 125–500 ng/mg). These concentrations declined with increasing distance from the implantation site (Fig. 2). Nonetheless, even at the most anterior and posterior extents of the ipsilateral hemisphere, significant amounts of mitoxantrone were detected at all time points. Mitoxantrone concentrations in the contralateral hemisphere were decreased approximately 10-fold. Overall, concentrations were maximal after 3 days and decreased slightly at each proceeding time point. After 35 days, significant amounts of mitoxantrone were still detectable.

In Vivo Intracranial Efficacy

In all the efficacy experiments, treatment with mitoxantrone wafers resulted in significantly improved survival. In our first efficacy study, compared with animals in the control group (median survival 18.5 days), rats in the 1% mitoxantrone wafer group had a median survival of 25 days (p < 0.0001) and those in the 5% mitoxantrone wafer group had a median survival of 33 days (p < 0.0001). The experiment was repeated with an additional group of rats receiving 10% mitoxantrone wafers. In the second experiment, the control group had a median survival of 22 days compared with that of animals receiving 1% mitoxantrone (median survival not reached, p < 0.0127), 5% mitoxantrone (median survival not reached, p < 0.0068), or 10% mitoxantrone (median survival not reached, p < 0.0073). In the last experiment, rats in the control group had a median survival of 19.5 days compared with that of animals receiving 1% mitoxantrone (median survival 31.5 days, p < 0.0003), 5% mitoxantrone (median survival 31.5 days, p < 0.0012), or 10% mitoxantrone (median survival 40 days, p < 0.0023). These data were combined with the following results: controls (23 rats, median survival 19 days), 1% mitoxantrone (23 rats, median survival 30 days, p < 0.0001), 5% mitoxantrone (24 rats, median survival 34 days, p < 0.0001), 10% mitoxantrone (16 rats, median survival 50 days, p < 0.0001; Fig. 3 and Table 2). No animal died without evidence of significant tumor growth.

Discussion

Mitoxantrone possesses in vitro activity against many tumor cell lines, and malignant gliomas are particularly sensitive to its effects. In a recent metaanalysis of in vitro glioma cell chemosensitivity, mitoxantrone was designated as one

Distance from polymer insertion site (mm)	Concentrations over ipsilateral coronal sections (ng/mg of brain tissue)			
	Day 3	Day 7	Day 18	Day 35
+3 - +5	325.8	247.4	128.5	90.3
+1 - +3	500.6	544.7	179.3	313.1
+1 - -1	849.9	556.1	270.6	505.9
-1 - -3	702.4	692.6	124.8	125.8
-3 - -5	228.8	261.7	121.4	85.1
-5 - -7	171.8	258.9	237.6	26.4

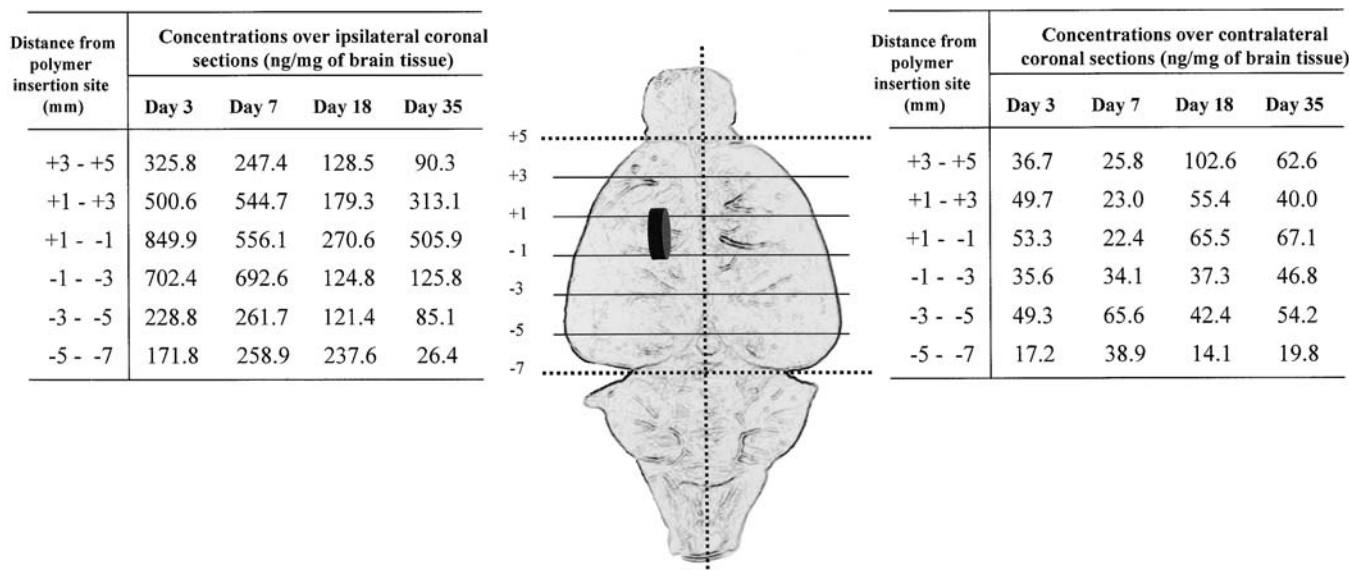| Distance from polymer insertion site (mm)	Concentrations over contralateral coronal sections (ng/mg of brain tissue)			
	Day 3	Day 7	Day 18	Day 35
+3 - +5	36.7	25.8	102.6	62.6
+1 - +3	49.7	23.0	55.4	40.0
+1 - -1	53.3	22.4	65.5	67.1
-1 - -3	35.6	34.1	37.3	46.8
-3 - -5	49.3	65.6	42.4	54.2
-5 - -7	17.2	38.9	14.1	19.8

FIG. 2. Tables and diagram demonstrating intracerebral distribution of mitoxantrone in the rodent. Following intracranial implantation of 10% mitoxantrone pCPP:SA wafers, rats were killed on Days 3, 7, 18, and 35. Brains were harvested, divided in the midsagittal plane, and then 2-mm-thick sections of each hemisphere were made in the coronal plane (center). Concentrations of mitoxantrone for each section at different time points are tabulated for the ipsilateral (left) and contralateral (right) hemispheres.

of the most potent drugs with a median lethal concentration that was 10 times less than that of carmustine, the most commonly used drug in the treatment of malignant brain tumors.[20] The mechanism of action by which mitoxantrone exerts its antitumor effects remains unclear, however. There is evidence that mitoxantrone binds DNA in tumor cells by a nonintercalative, electrostatic interaction and induces both protein-associated and nonprotein-associated DNA strand scissions.[1,3] It has also been suggested that mitoxantrone acts through the trapping of the nuclear enzyme DNA topoisomerase II as a covalent complex on DNA.[11] In addition, mitoxantrone has demonstrated strong antiangiogenic properties.[13]

Nonetheless, the in vitro potency of mitoxantrone has not translated into significant clinical success in the treatment of malignant gliomas. Data from a phase II study conducted in patients harboring Grade III or IV gliomas failed to demonstrate any efficacy following systemic mitoxantrone therapy.[18] Data from postmortem studies of patients treated with mitoxantrone have revealed poor penetration of mitoxantrone across the blood–brain barrier,[17] which in part explains the lack of clinical efficacy. Systemic treatments are also limited by dose-dependent myelosuppression and cardiotoxicity.[10] Indeed, the effectiveness of many chemotherapeutic agents for CNS malignancies depends on limitations imposed by both the blood–brain barrier, which restricts therapeutic drug quantities from reaching the brain, and adverse systemic toxicities.

To achieve therapeutic concentrations of mitoxantrone within the CNS while minimizing systemic exposure, we devised a strategy based on local delivery. Given that 80% of malignant brain tumors recur within 2 cm of the original tumor site[12,16] and that metastases are exceedingly rare, strategies in improving local tumor control are very promising. This local delivery strategy has been implemented clinically with carmustine-loaded pCPP:SA wafers and has demonstrated efficacy in patients with both recurrent[5,6] and newly diagnosed[4,19] malignant gliomas. Furthermore, it has been previously shown that direct intracerebral infusion of mitoxantrone in patients with glioblastoma multiforme is well tolerated.[2]

We first confirmed the in vitro potency of mitoxantrone against both rodent and human glioma cell lines. The sensitivity profile of the 9L gliosarcoma cell line mimicked that of the least sensitive human glioma cell line (U251). Based
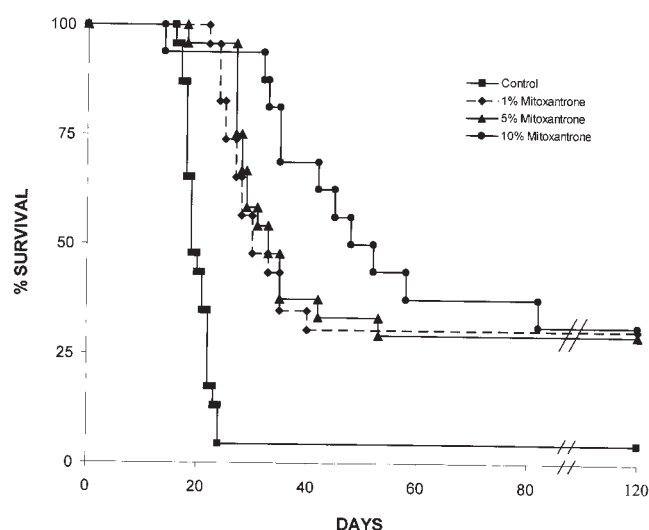

FIG. 3. Graph demonstrating Kaplan–Meier survival curves for combined data from three efficacy experiments (Table 2). Fischer 344 rats were intracranially injected with 9L gliosarcoma cells and 5 days later underwent implantation of mitoxantrone pCPP:SA wafers (0, 1, 5, or 10% mitoxantrone).

on these findings we decided to use the well-established intracranial 9L gliosarcoma in our in vivo studies.

Next, we fashioned implantable wafers composed of mitoxantrone and the biodegradable polymer pCPP:SA. The in vitro release was rapid for all drug-loading percentages due to the solubility of mitoxantrone dihydrochloride in aqueous solutions. This release profile, however, reached therapeutic concentrations of mitoxantrone throughout the rat brain for up to 35 days after wafer implantation (Fig. 2). The extended concentrations of mitoxantrone could be due to either decreased elimination of the drug from the CNS or more prolonged release from the wafer implant than that observed in our in vitro release study. The inability of mitoxantrone to penetrate the blood–brain barrier likely prevents access to the systemic circulation, thereby limiting elimination from the CNS. Moreover, in our in vitro studies we could have overestimated the rate of mitoxantrone release from the pCPP:SA matrix by approximating the volume of distribution of the rodent brain as an infinite sink condition. Thus, the in vivo release of mitoxantrone into brain parenchyma is probably less rapid and more prolonged than that predicted in our in vitro experiments. The sustained therapeutic concentrations of mitoxantrone achieved in the rodent brain with our local delivery strategy resulted in the improved survival of tumor-bearing animals in our in vivo efficacy studies. Due to the small size of the rodent brain, interpretation of these results with regard to clinical situations is difficult. Future studies with more complex animals and more precise techniques, such as quantitative autoradiography, are needed to determine better the distribution of locally delivered mitoxantrone within the brain.

Based on our intracranial toxicity study, we decided to use the two lower dose mitoxantrone wafer formulations, 1 and 5%, for our initial efficacy study. Survival was significantly prolonged for both treatment groups in this study. Then, because both the 1 and 5% mitoxantrone wafers were well tolerated (no animal treated with 1 or 5% wafers died before the control animals and none without significant tumor), we decided to add another treatment group (10% mitoxantrone) to our second efficacy study. This experiment confirmed the efficacy and lack of toxicity of the mitoxantrone wafers. Treatment with mitoxantrone resulted in significantly prolonged survival and did not produce any early deaths in 22 animals. Note that one of the seven control animals survived through the length of the study. Although this did not affect the significance of the experiment, we decided to repeat the study. This final efficacy experiment also resulted in prolonged survival in treated animals, confirming the results of the two prior studies.

Overall, in each experiment, there was a statistically significant prolongation of survival for all groups treated with mitoxantrone wafers. Higher drug loading resulted in improved survival. On average, implantation of 1% mitoxantrone wafers prolonged survival by 58%, 5% mitoxantrone by 79%, and 10% mitoxantrone by 141%. Moreover, each mitoxantrone loading dose produced long-term survivors, that is, animals surviving longer than 120 days: 30% long-term survivors with 1% mitoxantrone; 29% with 5% mitoxantrone; and 31% with 10% mitoxantrone.

Unfortunately, there is no existing animal tumor model that perfectly replicates the characteristics of high-grade brain tumors in humans. The 9L gliosarcoma is less invasive than most gliomas in humans. Furthermore, treatment

TABLE 2

In vivo efficacy of mitoxantrone wafers in the treatment of intracranial 9L gliosarcoma in Fischer 344 rats

Experiment No.	Treatment	No. of Rats	Median Survival (days)	No. of Long-Term Survivors*	p Value†
1	control	8	18.5	0	
	mitoxantrone				
	1%	8	25	1	<0.0001
	5%	8	33	0	<0.0001
2	control	7	22	1	
	mitoxantrone				
	1%	7	NR	4	0.0127
	5%	8	NR	4	0.0068
	10%	8	NR	4	0.0073
3	control	8	19.5	0	
	mitoxantrone				
	1%	8	31.5	2	0.0003
	5%	8	31.5	3	0.0012
	10%	8	40	1	0.0023
total	control	23	19	1	
	mitoxantrone				
	1%	23	30	7	<0.0001
	5%	24	34	7	<0.0001
	10%	16	50	5	<0.0001

* Long-term survivors defined as those animals surviving longer than the duration of the experiment (120 days). Abbreviation: NR = not reached.
† Log-rank test.

occurred just 5 days after tumor inoculation when the tumor burden was small. Thus, these results and their application to clinical situations should be interpreted with some caution.

Conclusions

In summary, we have developed a delivery system for mitoxantrone which achieves sustained therapeutic drug concentrations in brain parenchyma while minimizing systemic exposure. This system circumvents the limitations imposed by the blood–brain barrier, thereby enabling us to exploit the antitumor potency of mitoxantrone. This strategy consistently prolonged survival in animals bearing malignant brain tumors. Nonetheless, future studies of mitoxantrone should be directed toward further investigation of the CNS toxicity associated with local delivery. Studies in more complex animals are needed to define maximally tolerated intracerebral drug concentrations more fully, and those concentrations should then be used to delineate the optimal dosage of mitoxantrone for local delivery. Based on our initial findings, locally delivered mitoxantrone is a strong candidate for potential clinical application in the treatment of malignant gliomas.

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