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Local Delivery of Doxorubicin for the Treatment of Malignant Brain Tumors in Rats

MACIEJ S. LESNIAK[1], URVASHI UPADHYAY[2], RORY GOODWIN[2], BETTY TYLER[2] and HENRY BREM[2]

[1]*Division of Neurosurgery, The University of Chicago Pritzker School of Medicine, 5841 S. Maryland Ave-MC3026, Chicago, Illinois 60637;*
[2]*Departments of Neurosurgery and Oncology, The Johns Hopkins University School of Medicine, Hunterian 817, 725 N. Wolfe St, Baltimore, Maryland 21205, U.S.A.*

Abstract. *Background: Local delivery of carmustine (BCNU) via biodegradable polymers has been shown to improve survival in patients with glioblastoma multiforme (GBM). In the current study, we hypothesized that local delivery of an anthracycline antibiotic, doxorubicin (DOX), might act to improve the survival of animals bearing experimental intracranial glioma. Materials and Methods: Polyanhydride polymers (PCPP-SA), containing either 3% or 5% ADR by weight, were prepared using the mix-melt method. Forty male Fisher 344 rats received an intracranial challenge with a lethal dose of 9L gliosarcoma cells. Five days later, they received DOX or blank polymer. There were a total of four treatment groups: 1) blank polymer; 2) 3% DOX polymer; 3) 5% DOX polymer, and 4) control group with no polymer. Results: Compared to control animals treated with no polymers or blank polymer, animals receiving DOX had significantly extended survival. The median survival for the control group was 21 days vs. 34 days ($p<0.01$) for the 3% DOX group and 45 days ($p<0.0001$) for the 5% DOX group. Conclusion: Doxorubicin, when delivered locally, is an effective monotherapeutic agent against experimental intracranial glioma.*

The field of biodegradable polymer drug release has improved the treatment of malignant gliomas, and sparked interest in the treatment of metastatic brain tumors, by allowing the local delivery of chemotherapy to brain tumors (4, 5, 27, 29-31). Phase III clinical trials utilizing carmustine (BCNU)-polymer combinations have shown Gliadel® to be efficacious in the treatment of malignant gliomas, both in the recurrent setting as well as at the time of initial surgery (27-31). The delivery of chemotherapeutic agents directly to the tumor bypasses the blood-brain barrier, thereby allowing a high concentration of drug at the site of interest while eliminating the toxicity associated with the traditional systemic delivery of chemotherapeutic drugs.

While different strategies to improve the efficacy of polymer-mediated chemotherapy against malignant gliomas or even metastatic brain tumors are currently being pursued, one potential strategy is to test drugs other than BCNU. One such drug is doxorubicin (DOX), which is currently used in the treatment of a wide variety of cancers, such as acute lymphoblastic leukemias, lymphomas, multiple myeloma, sarcomas, mesotheliomas, germ cell tumors of the ovary or testis, carcinomas of the head and neck, and breast (2). Doxorubicin is an anthracycline antibiotic produced by the *Streptomyces peucetius varieta caesius*. Doxorubicin blocks DNA and RNA synthesis by inhibiting topoisomerase II (3).

Despite the clinical effectiveness of DOX in the treatment of many malignant tumors, clinical trials involving systemic administration of the drug have demonstrated very limited efficacy in the treatment of gliomas (11, 21). Very high doses of DOX must be administered systemically to exert any therapeutic benefit and these doses are highly neurotoxic and, therefore, ineffective in treating central nervous system (CNS) malignancies (15, 17). The limited efficacy of DOX when administered systemically can be explained by the poor penetration of the drug through the blood-brain barrier and the effect of p-glycoprotein-mediated efflux from the cerebrospinal fluid (CSF) (15, 17, 18). The low lipophilicity and high molecular weight of DOX essentially prevent the delivery of the agent across the blood-brain barrier.

Correspondence to: Maciej S. Lesniak, MD, Division of Neurosurgery, The University of Chicago Pritzker School of Medicine, 5841 S. Maryland Ave-MC3026, Chicago, Illinois 60637, U.S.A. Tel: (773) 834-4757, Fax: (773) 834-2608, e-mail: mlesniak@surgery.bsd.uchicago.edu

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Recent *in vitro* data demonstrate that DOX is toxic to glioblastoma cell lines (1, 6, 24). Moreover, *in vivo* data in animal models of malignant glioma suggest that DOX is an effective anti-glioma agent (13, 14, 16, 19, 20, 22, 24, 25). In fact, in a recently published clinical study, ten patients with grade III or IV gliomas were treated with direct DOX infusion *via* an Ommaya reservoir (28). Fifty percent of the patients showed objective radiologic response. There were no clinically significant adverse reactions, either in the brain or systematically. Intra-tumoral administration of DOX, therefore, appears to be a safe and potentially effective treatment and should be further explored in the management of brain gliomas resistant to conventional forms of treatment.

In this study, we hypothesized that local intracerebral delivery of doxorubicin *via* a polymer matrix might be beneficial in the treatment of malignant glioma. Specifically, we investigated: i) the efficacy of DOX against an experimental brain tumor cell line *in vitro*; ii) the drug release kinetics of implantable wafers composed of DOX and the biodegradable polymer pCPP:SA; iii) the toxicity of the DOX-polymer system after implantation into the rat brain; iv) the ability of DOX delivered *via* the polymer system *in vivo* to extend survival in an intracerebral malignant glioma model.

Materials and Methods

Tumor cell lines. 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, USA). The cells were maintained in tissue culture in Dulbecco's minimum essential medium with 10% fetal bovine serum, streptomycin (80.5 μg/ml), penicillin (base; 80.5 units/ml) and 1% L-glutamine (all products from GIBCO laboratories, Grand Island, NY, USA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C. The cells were grown to confluence, detached with 0.25% trypsin in Dulbecco's phosphate-buffered saline and resuspended in medium.

In vitro activity of DOX. Inhibition of tumor proliferation was tested with rodent 9L glioma. Cells were plated at 10,000 cells/well in 24-well plates with increasing concentrations of DOX, ranging from 1 ng/ml to 50 μg/ml. The cells were counted after 5-day exposure by using a cell counter and compared with control cells receiving no DOX.

Wafer preparation. The matrix pCPP:SA had a 10:90 molar ratio. DOX was combined with pCPP:SA (10:90), methanol to dissolve DOX and methylene chloride. Cylindrical wafers, weighing 10 mg each (3 mm in diameter, 1 mm thick), were then manufactured using the steel molding press.

Drug release kinetics. The pCPP:SA wafers were placed in 1.5 ml vials containing 1 ml of 0.1 M phosphate-buffered saline solution, ph 7.4. The vials were capped to prevent evaporation and placed in an incubator at 37˚C. The saline solution was removed and replaced to approximate infinite sink conditions. The amount of DOX released was determined by reading samples on the Bertramm Spectrophotometer at 480 nm and by calculating the drug concentration based on a standard curve.

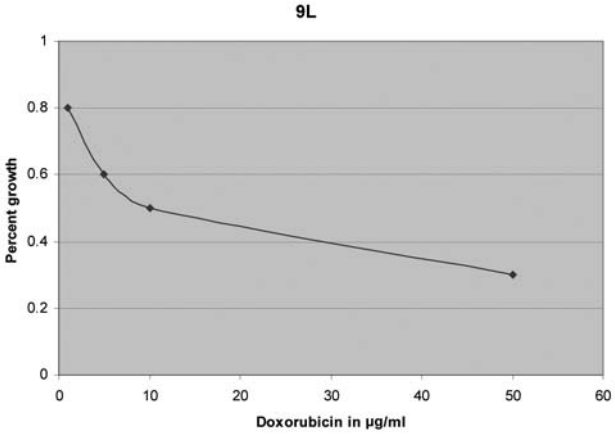

Figure 1. *In vitro growth inhibition of rodent 9L glioma. The cell line was incubated with doxorubicin for five days and shows a 10-50 ng/ml DOX tumoricidal range.*

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 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 $1x10^2$ 9L glioma cells were implanted. 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 1mm 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 tumor implantation.

In vivo DOX wafer toxicity. To determine the maximally tolerated DOX loading dose, 80 rats, evenly divided into 4 groups, underwent intracerebral implantation of wafers containing 10%, 7%, 5% and 3% DOX pCPP:SA wafers. The animals were closely monitored for signs of toxicity, including wound healing problems, weight loss, failure to thrive and neurological deficits. Survival was then assessed and autopsies performed on all animals.

Table I. *Toxicity study.*

Polymer % Dox	Animals alive (#, %, N=20)	Early deaths (1-14 days) (#, %, N=20)	Late deaths (14 days+) (#, %, N=20)
10%	0	3, 15%	17, 85%
7%	9, 45%	1, 5%	10, 50%
5%	15, 75%	1, 5%	4, 20%
3%	20, 100%	0, 0%	0, 0%

Dox: Doxorubicin

Table II. *Histopathological analysis of toxicity associated with doxorubicin.*

Polymer (%DOX)	Hemorrhage	Necrosis	Edema
Early deaths (1-14 days)			
10% (n=3)	+++	+	++
7% (n=1)	++	+	++
5% (n=1)	++	+	++
Late deaths (14+ days)			
10% (n=17)	+	+++	+++
7% (n=10)	+	+++	+++
5% (n=4)	–	+	++

DOX: Doxorubicin
+++ Present >5 mm from tumor/polymer
++ Present <5 but >2 mm from tumor/polymer
+ Present <2 mm from tumor/polymer

Histological evaluation. In the toxicity study, the brains, lungs, hearts, kidneys, livers, spleens and intestines were all examined when an animal died following implantation of a polymer. The same protocol was repeated when an animal died in the efficacy group. In addition, representative animals from each efficacy group were euthanized on days 7 and 14 after polymer implant and the brains were removed and compared to non-treated controls. All tissue was fixed in 10% formalin, blocked in paraffin and stained with hematoxylin and eosin (H&E).

Sources of supplies and equipment. The matrix pCPP:SA was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD, USA). DOX was purchased from Sigma-Aldrich Corp. (St. Loius, MO, USA). The ultraviolet/visible spectrophotometer (Genesys 5) used to determine the amount of DOX released was acquired from Spectronic (Rochester, NY, USA).

Animals. A total of 200 10-week-old, female Fisher 344 rats, weighing 180 to 220 g, were purchased from Charles River Laboratories (Wilmington, MA, USA). Eighty animals were used for toxicity studies and the remaining 120 were used for efficacy studies (40 animals/study, repeat experiment 3 times). The animals were kept in standard animal facilities with 3 or 4 rats per cage, and given free access to rat chow and water. They were housed in accordance with the policies and principles of laboratory care of the Johns Hopkins University School of Medicine Animal Care and Use Committee, USA.

Statistical methods. For *in vitro* studies, data was analyzed using the two-tailed Student's *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.

Results

In vitro efficacy of DOX. DOX is a potent inhibitor of rodent glioma cell growth. At a concentration of 50 ng/ml (range 10-50 ng/ml), 9L glioma was inhibited by at least 97.5% after 5 days of incubation with DOX (Figure 1).

Polymer formulation and drug release kinetics. DOX was loaded 1-10% by weight into pCPP:SA controlled release polymer and drug release was assayed by ultraviolet spectroscopy. Spectroscopy showed that while the 1% loaded polymer released 6.5% (65 μg) of drug over 200 hours, the 10% loaded polymer released 21% (210 μg) of drug over 200 hours, a concentration well in excess of the tumoricidal range (Figure 2).

In vivo DOX wafer toxicity. None of the animals exhibited any problems with wound healing or weight loss. Animals which developed toxicity uniformly exhibited failure to thrive, as manifested by inability to reach food and water. In the 10% and 7% doxorubicin groups, the majority of animals (>70%) also developed hemiparesis. Animals which were symptomatic for more than 24 hours were then euthanized. When 10% DOX pCPP:SA polymers were administered to F344 rats, all the animals died by day 40. On lowering the dosage to 7% DOX polymers, we found that 11 out of 20 rats died by day 100, while the remaining 9 survived to day 350. Five rats loaded with 5% DOX polymers died by day 150, while the remaining 15 survived to day 350. Of the 20 rats loaded with 3% DOX polymer, no toxicity-related deaths were seen. These data are presented in Table I.

Histopathological findings in toxicity study. All animals which died during the toxicity study were subjected to autopsies and their organs (brain, lung, heart, kidney, liver, spleen and intestine) were removed and examined. Analysis of brains during the time of early death (1-14 days after polymer implant) was remarkable for the presence of hemorrhage and edema. There was minimal amount of necrosis. However, the majority of late deaths (14 days +) appeared to be secondary

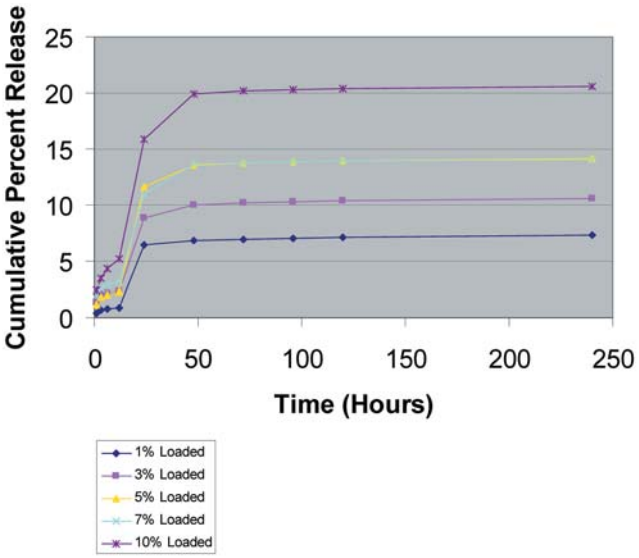

Figure 2. *In vitro release kinetics for 1-10% DOX in pCPP:SA polymer.*

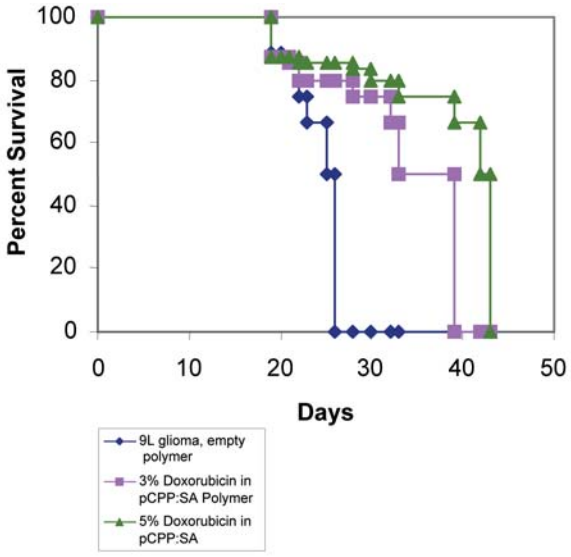

Figure 4. *Results of in vivo intracranial efficacy trials. Compared to control animals treated with no polymers or blank polymer, animals receiving DOX had significantly extended survival. The median survival for the control groups was 21 days vs. 34 days ($p<0.01$) for the 3% DOX group and 45 days ($p<0.0001$) for the 5% DOX group.*

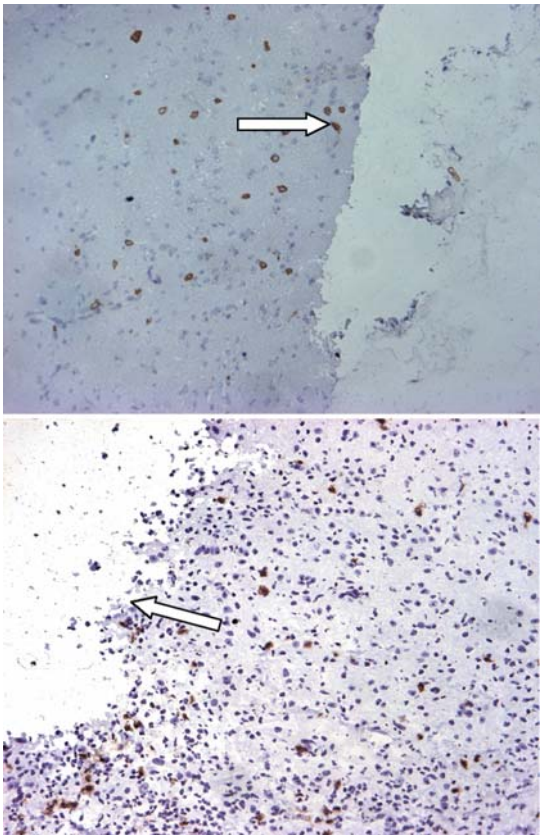

Figure 3. *Histological analysis of animal brains obtained in the toxicity study. The figure illustrates the presence of necrosis (arrow) which was frequently seen as a late (14 days +) cause of death following DOX-polymer implant.*

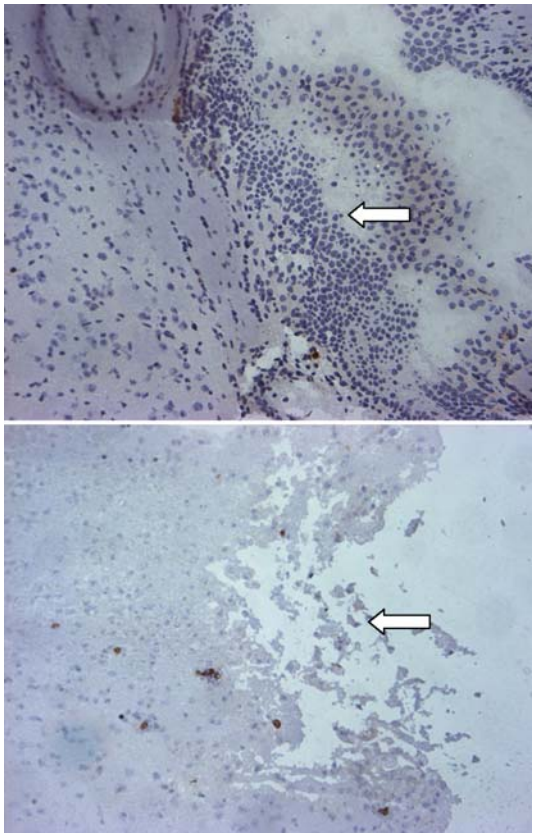

Figure 5. *Histological analysis of animal brain treated with DOX. Representative section from (A) control animal and (B) DOX-treated animal shows the presence of tumor cells (arrow in A) and tissue necrosis (arrow in B).*

to tissue necrosis and local edema (Figure 3). This data is summarized in Table II. The lungs, heart, liver, spleen, kidneys and intestine were normal without any associated hemorrhage, necrosis, or edema.

In vivo intracranial efficacy. Of the 40 F344 rats which received intracranial 9L implantation, the animals receiving empty pCPP:SA polymer showed a median survival time of 21 days. Those treated with DOX polymers showed prolonged survival time: for those loaded with 5% DOX- pCPP:SA polymer, median survival time was 45 days ($p<0.0001$), compared to 34 day ($p<0.01$) survival for those loaded with 3% DOX-pCPP:SA polymers (Figure 4).

Histopathological findings in efficacy study. In the efficacy study, all animals which died were subjected to autopsy and histological analysis. In the control group as well as DOX-treated groups, the cause of death was a large intracranial tumor. Representative animals from the DOX groups were also sacrificed on days 7 and 14 after polymer implantation. The brains treated with DOX showed significantly decreased tumor burden accompanied by local tissue necrosis. While the tissue necrosis occurred within the tumor, there was also evidence of necrosis in the surrounding brain (Figure 5). This necrosis was most evident in the 5% DOX group, and extended to within 4 mm of the tumor margin in 4/10 treated animals. Only one animal in the 3% DOX group showed evidence of brain necrosis, and this was limited to 2 mm of the tumor margin.

Discussion

DOX has been shown to inhibit tumor cell growth in numerous tumor lines and is currently used to treat a host of cancers from multiple myeloma to carcinoma of the head and neck. Previous studies have shown that DOX is toxic to glioblastoma cell lines (1, 24). We have shown that DOX is a potent inhibitor of rodent glioma *in vitro* and *in vivo*.

While the *in vitro* potency of DOX is remarkable and its current indications in treating peripheral tumors have proven efficacious, DOX has yet to be used successfully to treat malignant gliomas. Systemically administered DOX has shown poor penetration of the blood-brain barrier and attempts to improve drug delivery, *via* cerebrospinal infusion, have not been very successful (15). Underlying the failure of these attempts has been the fundamental limitation in achieving therapeutic concentrations of DOX in the CNS while minimizing systemic adverse side-effects.

To achieve a therapeutic concentration of DOX, we devised a strategy of delivering DOX locally and in a controlled manner. Since the majority of malignant brain tumors recur very close to the original tumor site, and given that these tumors are rarely metastatic, local delivery of antitumor agents represents an attractive approach (7, 12, 23). The strategy of local delivery is used clinically with carmustine-loaded pCPP:SA wafers and has demonstrated efficacy in patients with both recurrent and newly-diagnosed malignant gliomas (7).

In the preceding experiments, we first confirmed that DOX is an inhibitor of glioma cell lines. We found that DOX antagonized the growth of all cell lines when delivered at 10-50 ng/ml. We then chose to use the 9L line for our *in vivo* studies. We manufactured an implantable disk made from DOX and the biodegradable polymer pCPP:SA and measured its release profile. We found that anywhere from 6.5% to 21% of the DOX loaded into the polymer was released over several days, depending on the percent of DOX in the polymer. The inability of DOX to penetrate the blood-brain barrier made us confident that no DOX was able to leave the CNS and enter systemic circulation. Furthermore, our *in vitro* release kinetics study may have overestimated the rate of DOX release from the polymer matrix by approximating the volume of distribution of the rodent brain as an infinite sink condition (7). We can therefore conclude that the *in vivo* release of DOX from the pCPP:SA matrix is slower and longer in duration than our *in vitro* data suggests. Indeed, pharmacokinetic studies of various drug-polymer combinations utilizing different animal and human models support this notion (8-10, 26) and further confirm that the release kinetics are a function of the polymer matrix rather than the chemotherapeutic agent.

Our *in vivo* toxicity studies indicated that polymers loaded with 3% DOX were well tolerated by F344 rats. We then used these low-dose DOX wafer formulations for our *in vivo* efficacy studies and found that both the 3% and the 5% treatment groups significantly extended the survival of subjects implanted with 9L gliosarcoma intracranially. Specifically, we observed a dose-dependent trend in survival of animals treated with DOX wafers. When 3% DOX wafers were administered, survival extended by 52.6% compared to controls. When 5% DOX wafers were administered, survival was extended by 137%. However, given the higher rate of toxicity seen with 5% polymers, we favor further development of lower concentrations of DOX-polymer wafers.

While these results are rather promising, we caution that our experimental model has key limitations in application to high-grade brain tumors in humans. First, the 9L gliosarcoma is not a perfect model of human glioma because it less invasive. Secondly, we administered treatment to our animals 5 days after tumor implantation, when the tumor was arguably smaller than those seen in most clinical scenarios.

In spite of these qualifications, the clinical prospects of local delivery of DOX are promising. Currently, some patients diagnosed with malignant glioblastoma receive BCNU wafers at the time of surgery. Intraoperative delivery of the BCNU wafer has been shown to increase 6-month survival by 50% and to prolong overall survival from 23 to 31 weeks (5). While these results are significant, the prognosis for patients diagnosed with glioblastoma multiforme remains poor. The elegance of a local delivery system with a novel chemotherapeutic agent is therefore an attractive prospect.

In these studies, we have shown that DOX shows robust tumoricidal activity against human glioma tumor cells lines. However, the clinical use of DOX has been severely limited by its known toxicity. Furthermore, DOX has not been shown to reach therapeutic levels interstitially due to difficulty penetrating the BBB. These limitations further suggest that DOX may be best used when delivered locally as an adjunct to current therapeutic modalities.

Conclusion

In these studies, we designed a successful means of delivering DOX locally, which minimizes systemic side-effects and delivers DOX in a controlled, sustained fashion at therapeutic concentrations. We showed that DOX, when delivered in this manner, significantly prolonged survival of rodents bearing malignant brain tumors. While these initial findings point to the clinical application of DOX in the treatment of malignant gliomas, further work must first be done to investigate CNS toxicity associated with local delivery in more complex animal models.

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