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The role of minocycline in the treatment of intracranial 9L glioma

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This study was designed to explore the question of whether minocycline, a semisynthetic tetracycline shown to inhibit tumor-induced angiogenesis, could control the growth of the rat intracranial 9L gliosarcoma. Minocycline was tested alone and in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) *in vivo*. Treatment was started at the time of intracranial implantation of 9L gliosarcoma into male Fischer 344 rats, 5 days later, or after tumor resection.

Minocycline was delivered locally with a controlled-release polymer or systemically by intraperitoneal injection. Systemic minocycline did not extend survival time. Local treatment with minocycline by a controlled-release polymer implanted at the time of tumor implantation extended median survival time by 530% ($p < 0.001$) compared to treatment with empty polymer. When treatment was begun 5 days after tumor implantation, minocycline delivered locally or systemically had no effect on survival. However, after tumor resection, treatment with locally delivered minocycline resulted in a 43% increase in median survival time ($p < 0.002$) compared to treatment with empty polymer. Treatment with a combination of minocycline delivered locally in a controlled-release polymer and systemic BCNU 5 days after tumor implantation resulted in a 93% extension of median survival time compared to BCNU alone ($p < 0.002$). In contrast, treatment with a combination of systemic minocycline and BCNU did not increase survival time compared to systemic BCNU alone. These results demonstrate that minocycline affects tumor growth when delivered locally and suggest that minocycline may be a clinically effective modulator of intracranial tumor growth when used in combination with a chemotherapeutic agent and surgical resection.

KEY WORDS • brain tumor • angiogenesis • minocycline • BCNU • drug delivery • chemotherapy

MINOCYCLINE and other tetracycline derivatives, long used clinically for their antibiotic effects, have recently been shown to have potential benefit in the treatment of cancer.[12,23,27] Although the mechanism of this antitumor effect has not yet been definitively elucidated, the tetracyclines have been shown to inhibit eukaryotic mitochondrial protein synthesis[12] and to inhibit tumor angiogenesis,[25] the latter presumably due to inhibition of collagenase.[9]

The growth of solid tumors is dependent on angiogenesis;[2–5] in particular, endothelial proliferation is a pathological characteristic of malignant gliomas, which are among the most vascular of all solid tumors.[1] Consequently, with the use of tetracyclines to inhibit tumor-induced neovascularization and thus control tumor growth, it may be clinically feasible to prolong survival time of patients with primary brain tumors.

To determine whether minocycline can control brain tumor growth, we investigated the ability of this agent to improve survival in the rat 9L gliosarcoma brain tumor model. Minocycline was administered either systemically by intraperitoneal injection or locally by intracranial implantation of a controlled-release polymer. Treatment was started at the time of tumor implantation, 5 days later, or after tumor resection to determine whether tumor size and vascular status altered the effectiveness of minocycline.

Antiangiogenic agents, including minocycline, have been shown to potentiate the efficacy of standard anticancer therapies for some extracranial malignancies.[23,27] To explore this possibility for brain tumors, we studied the effect of systemic or local minocycline therapy alone and in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on survival of rats with intracranial 9L gliosarcoma.

Materials and Methods

Study Design

Two hundred thirty-four male Fischer 344 rats, weighing approximately 200 g each, were anesthetized with an intraperitoneal injection of 3 to 5 ml/kg of a stock solution containing ketamine

hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol in 0.9% NaCl. The 9L gliosarcoma was then surgically implanted within their brains.

The animals were treated either at the time of tumor implantation or 5 days later, which allowed time for the tumors to become established, vascularized masses.

For animals treated on the day of tumor implantation, there were four treatment groups of 10 rats each: 1) minocycline, 70 mg/kg daily in two divided doses given intraperitoneally with blank polymer implanted intracranially; 2) minocycline, 100 mg/kg daily in two divided doses given intraperitoneally with blank polymer implanted intracranially; 3) minocycline-loaded polymer implanted intracranially with two daily intraperitoneal injections of 0.05 M Trizma carrier solution;* and 4) blank polymer implanted intracranially and two daily injections of 0.05 M Trizma carrier solution intraperitoneally (control animals).

Three experiments with animals treated on Day 5 after tumor implantation were conducted (Table 1). Experiment 1, which was designed to assess the effects of interstitial minocycline on survival when implanted intracranially after surgical resection, had two treatment groups: 1) surgical resection with implantation of empty polymer within the resection cavity, and 2) surgical resection with implantation of minocycline polymer. Experiment 2, which assessed the benefits of combining interstitial minocycline with systemic BCNU therapy 5 days after tumor implantation, had four treatment groups: 1) minocycline polymer implanted intracranially within the tumor mass, no systemic BCNU; 2) empty polymer implanted intracranially and intraperitoneal BCNU; 3) minocycline polymer implanted intracranially and intraperitoneal BCNU; and 4) empty polymer implanted intracranially and no systemic BCNU (control animals). Experiment 3, which investigated the potential benefit of systemic minocycline as an adjunct to standard systemic BCNU therapy, had three treatment groups: 1) a single dose of BCNU intraperitoneally and injections of minocycline (75 mg/kg in two divided doses every day) in 0.05 M Trizma carrier solution; 2) a single dose of BCNU intraperitoneally and two daily injections of 0.05 M Trizma solution without minocycline; and 3) two daily injections of 0.05 M Trizma solution without minocycline or BCNU (control animals).

All animals were kept in standard facilities and given free access to food and water. The rats were examined twice daily after surgery. Particular attention was given to behavioral changes and neurological deficits. No neurological effects attributable to the locally delivered minocycline were observed. At the time of death, the brains were removed from all animals, fixed in 10% formalin for at least 1 week and prepared for hematoxylin and eosin staining. Sections through the tumor were stained to verify the presence of neoplastic tissue.

Tumor Line

The 9L gliosarcoma† was maintained subcutaneously as solid masses in the flanks of male Fischer 344 rats. The tumor in carrier rats was passaged every 2 to 3 weeks.

Polymer Preparation

Ethylene vinyl acetate[13] (EVAc; 40% vinyl acetate by weight) was washed extensively in absolute ethyl alcohol, with total volume changes every 24 hours to extract the inflammatory antioxidant butylhydroxytoluene, which was monitored spectrophotometrically at 230 nM in the washes. When the absorbance fell below 0.03 U, the polymers were dried in a vacuum desiccator for 5 days.[13]

Minocycline was incorporated into the polymer matrix by a modification of the fabrication procedure described by Rhine, *et al.*[19] The drug was ground with a glass pestle through a 200-mesh (74-μm) screen in a tissue sieve, and suspended in a 10% solution of EVAc and methylene chloride. The EVAc solution with or without minocycline was poured into glass cylindrical molds and cooled to −70°C. After 20 minutes, the solidified polymers were removed, transferred to a −30°C freezer, and allowed to dry for 2 to 3 days. The polymers were then dried in a vacuum desiccator for 3 to 4 days, and stored at −20°C. The final minocycline concentration in the polymer was 50% by weight. Polymers without drug were prepared as controls.

For the intracranial experiments, the polymers were cut into disks measuring 3 × 3 × 1 mm. The average weight of the polymers loaded with 50% minocycline for the intracranial experiments was 11.3 ± 0.4 mg. Thus, the total minocycline dose implanted was 5.65 ± 0.2 mg per animal. Before implantation, all polymers were placed under ultraviolet light for 1 to 2 hours.

TABLE 1

*Treatment initiated 5 days after tumor implantation**

Treatment Group	No. of Rats	Surgical Resection	Intracranial (IC) Implant	Intraperitoneal (IP) Injections
Experiment 1:				
surgery only	18	yes	empty EVAc	none
surgery/mino (IC)	17	yes	50% mino-EVAc	none
Experiment 2:				
mino (IC)	8	no	50% mino-EVAc	carrier solution
BCNU (IP)	22	no	empty EVAc	14 mg/kg BCNU × 1
mino (IC) BCNU (IP)	23	no	50% mino-EVAc	14 mg/kg BCNU × 1
controls	17	no	empty EVAc	carrier solution
Experiment 3:				
mino (IP)/ BCNU (IP)	10	no	none	14 mg/kg BCNU × 1 mino 75 mg/kg/day
BCNU (IP)	10	no	none	14 mg/kg BCNU × 1 carrier solution daily
controls	10	no	empty EVAc	carrier solution daily

* Mino = minocycline; BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; EVAc = ethylene vinyl acetate copolymer.

Intracranial Tumor Implantation and Treatment

The 9L gliosarcoma from carrier rats was cut into 1-mm^3 pieces for intracranial implantation. After induction of anesthesia, the head of each rat was shaved and prepared with 70% ethyl alcohol and povidone-iodine, 10% solution. A midline incision was made by means of microsurgical technique and the coronal and sagittal sutures were identified. A burr hole 3 mm in diameter was made, centered on a point 5 mm posterior to the coronal suture and 3 mm lateral to the sagittal suture. The dura was opened in a cruciate fashion by means of a Weck microsurgical knife. The cortex and underlying white matter were resected with suction until the brainstem was visualized. The intracerebral surgical site was irrigated with sterile 0.9% NaCl until clear, and the tumor piece was placed in the depths of the cortical resection. For animals treated at the time of tumor implantation, a minocycline polymer or empty polymer was placed into the cortical defect next to the tumor piece. The skin was closed with surgical staples. The rats treated with systemic minocycline on the day of tumor implantation were given the first dose after skin closure.

Delayed Treatment Without Surgical Resection

The animals underwent reoperation for treatment beginning 5 days after tumor implantation. They were anesthetized and prepared

* Minocycline and Trizma carrier solution supplied by Sigma Chemical Co., St. Louis, Missouri.

† Gliosarcoma (9L) supplied in 1985 by Marvin Barker, Brain Tumor Research Center, University of California, San Francisco, California.

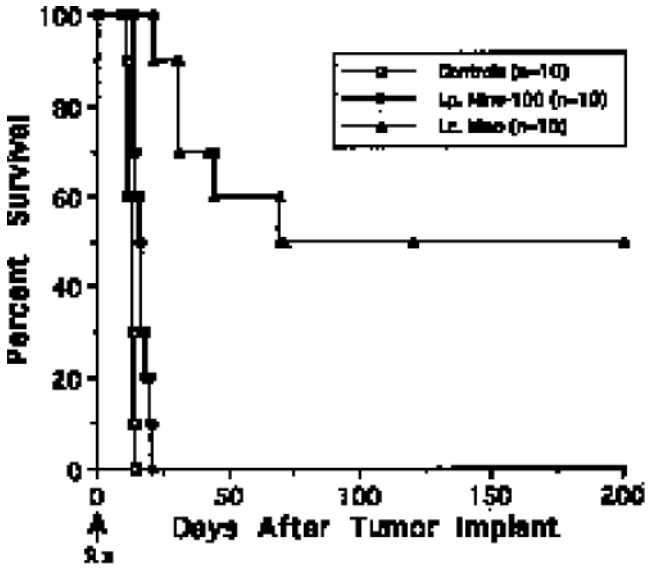

FIG. 1. Graph illustrating survival curves for animals treated at the time of tumor implantation. i.p. Mino = intraperitoneal minocycline; i.c. Mino = intracranial minocycline; Rx = time of initiation of treatment.

as previously described; the midline incision was reopened and the burr hole identified. An empty or minocycline-loaded polymer was placed next to the tumor in the previously created intracerebral defect. The surgical field was irrigated with sterile 0.9% NaCl until all bleeding had stopped, and the skin was again closed with staples. Drug injections were administered intraperitoneally after skin closure.

Delayed Treatment With Surgical Resection

Five days after tumor implantation, 35 animals underwent reoperation for surgical resection of the tumor mass. After induction of anesthesia, the skin staples were removed and the scalp incision reopened, thus exposing the burr hole. The tumor was resected microsurgically with suction until the brainstem was again visualized. The bleeding was controlled by irrigation with 0.9% NaCl and by packing with Gelfoam, which was removed before polymer implantation. Controlled-delivery polymer loaded with minocycline (50% by weight) or empty polymer was implanted into the tumor cavity after the bleeding stopped. The incision was then reclosed with skin staples and intraperitoneal drug administration was initiated.

Systemic Drug Administration

Carmustine (BCNU) was reconstituted according to the manufacturer's instructions by first dissolving 100 mg of powder in 3 ml of sterile anhydrous ethanol, then adding 27 ml of sterile water for injection. This yielded a solution of 3.3 mg/ml BCNU in 10% ethanol, which was administered as a single dose of 14 mg/kg by intraperitoneal injection at the time of polymer implantation. This is the best reported protocol for systemic treatment of the 9L gliosarcoma in rats.[20,21]

Minocycline was dissolved in a 0.05 M Trizma solution, pH 8.4. Animals were treated with a dose of minocycline consisting of 70, 75, or 100 mg/kg/day, as described above, divided in two daily 1-ml intraperitoneal injections. Control animals and animals receiving minocycline-loaded polymer were given 1-ml injections of the 0.05 M Trizma solution alone.

Release Kinetics

To characterize the release of minocycline from EVAc, two polymers, 50% loaded with minocycline, were each placed in 5 ml of Dulbecco's phosphate-buffered solution and incubated at 37°C. At specific times after beginning the incubation (1, 3, 6, 18, and 28 hours, then daily for 8 weeks, and then approximately every other day until Day 90), the buffer was replaced with fresh buffer. The discarded buffer was frozen and stored at −30°C for measurement of minocycline.

Minocycline levels were measured by spectrophotometric absorbance at a wavelength of 324 nm. A standard curve was constructed and the minocycline levels in the samples were determined. The amount of minocycline released between collection points was used to calculate cumulative release.

Statistical Analysis

The Kruskal–Wallis and Mann–Whitney nonparametric tests were used for analysis of variance. The Newman–Keuls' multiple-range nonparametric analog for multiple comparisons was used to determine differences between treatment groups.[33]

Results

Efficacy of Minocycline: Monotherapy

When treatment began at the time of tumor implantation, minocycline delivered intracranially with a 50% loaded polymer extended median survival from 13 days in control animals to 69 days (530%; $p < 0.001$; Fig. 1). Furthermore, half the animals were still alive 100 days after tumor implantation. Systemically administered minocycline at 70 mg/kg/day did not affect survival, but increasing minocycline to 100 mg/kg/day extended median survival by 3 days (23%; $p < 0.05$; Fig. 1). However, at 100 mg/kg/day minocycline, animals lost more than 20% of their body weight over the course of treatment (2 to 3 weeks), suggesting generalized toxicity. By contrast, the group of animals implanted with polymer containing minocycline gained approximately 8% of their body weight during the same treatment period. When treatment (intracranially with a 50% loaded polymer or systemically at 100 mg/kg/day) was started 5 days after tumor implantation, minocycline did not increase median survival time.

By surgically resecting the tumor, we were able to decrease the tumor burden and disrupt the vascular supply. After tumor resection, treatment with intracranial minocycline by polymer extended median survival time by 43% compared to control animals implanted with empty polymer after tumor resection ($p < 0.002$; Fig. 2 *left*).

Efficacy of Minocycline in Combination With BCNU

To determine whether minocycline was able to potentiate the efficacy of chemotherapy, combinations of minocycline, local or systemic, and BCNU were tested against established 9L gliosarcoma. With treatment beginning 5 days after tumor implantation, minocycline delivered intracranially in a 50% loaded controlled-release polymer in combination with a single dose of systemic BCNU (14 mg/kg) significantly increased median survival by 93% compared to systemic BCNU alone ($p < 0.002$) and by 330% compared to controls ($p < 0.001$). Systemic BCNU (14 mg/kg) increased median survival time by 123% compared to controls ($p < 0.05$). Locally delivered minocycline alone did not affect median survival time when treatment began on Day 5. These results are shown in Fig. 2 *center*. When systemic minocycline

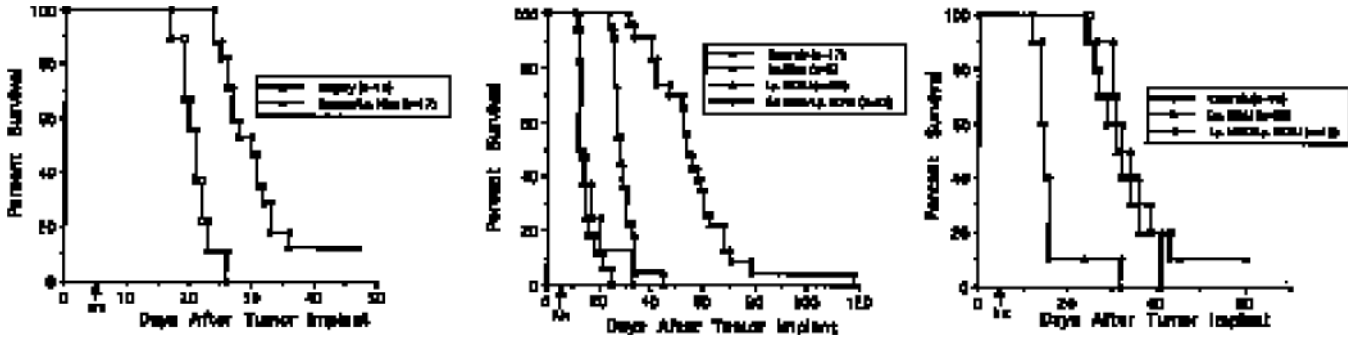

FIG. 2. Graphs illustrating survival curves for three treatment groups. *Left:* Animals treated 5 days after tumor implantation with surgical resection and implantation of ethylene vinyl acetate copolymer, with or without minocycline (mino). The study design is outlined in Table 1, Experiment 1. i.c. = intracranial; Rx = treatment day. *Center:* Animals treated 5 days after tumor implantation with i.c. mino polymer implantation and/or systemic 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). The study design is outlined in Table 1, Experiment 2. i.p. = intraperitoneal. *Right:* Animals treated 5 days after tumor implantation by i.p. BCNU with or without systemic mino. The study design is outlined in Table 1, Experiment 3.

was given in combination with systemic BCNU, there was no additional increase in survival time compared to animals treated with BCNU alone (Fig. 2 *right*).

Histological Analysis

A histological analysis of brain specimens confirmed the presence of tumor in all animals at the time of death. There was no apparent difference in tumor size or histology between treatment groups and controls. No evidence of hemorrhage or infection was seen.

Release Kinetics

Minocycline continued to be released from EVAc *in vitro* for 90 days (Fig. 3). Over the first 24 hours, minocycline released approximately 12% of the drug load. Over the next 2 weeks, the rate of release was approximately 1% of the total drug load per day, which then decreased to between 0.4% and 0.6% of the total drug load per day.

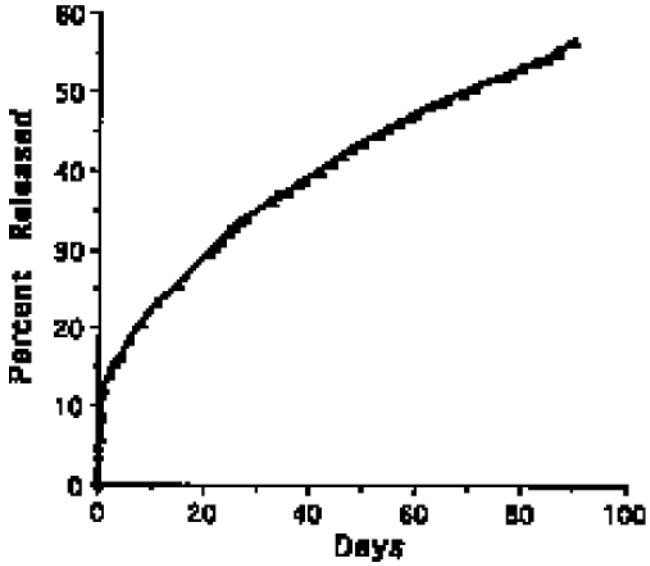

FIG. 3. Graph illustrating cumulative release of minocycline from 50% loaded ethylene-vinyl acetate (by weight) into phosphate-buffered saline over 90 days.

Discussion

The potential benefits of administration of minocycline in the treatment of intracranial tumors in the rat 9L gliosarcoma model were investigated in this study. In these animals, minocycline prolonged survival only when administered interstitially in a controlled-release polymer at the time of tumor implantation or at the time of tumor resection. No effect was seen on established tumor masses without concurrent surgical resection, suggesting that a cytoreductive treatment is required for minocycline to be of therapeutic benefit.

We tested this hypothesis by combining minocycline with the cytotoxic agent BCNU for treatment of established intracranial tumors without surgical resection. Locally delivered minocycline doubled the length of survival achieved with BCNU alone. In contrast, systemically delivered minocycline did not increase survival time, presumably because of inadequate drug delivery to the tumor.

Antitumor Effects of Tetracyclines

Tetracyclines have long been used clinically for their broad-spectrum antimicrobial properties, which result from inhibition of protein synthesis due to binding to the bacterial 30S ribosome.[22] These compounds have also been shown to inhibit tumor growth in a variety of experimental cancer models *in vivo*, including a rat Leydig cell tumor,[30] a rat mammary gland tumor,[12] human renal cell carcinoma xenografts in Syrian hamsters[12] and nude rats,[29] the rat Walker 256 sarcoma,[31] the rabbit VX2 carcinoma,[25] and the Lewis lung carcinoma in mice.[23,27]

There is evidence that the inhibition of tumor progression achieved with the tetracyclines is related to inhibition of mitochondrial protein synthesis in the neoplastic cells,[12,29–31] resulting in G_1 phase growth arrest;[31] however, another recently discovered pharmacological property of

the tetracyclines is their ability to inhibit extracellular matrix metalloproteinases, including collagenase.[6] Because solid tumor growth is angiogenesis dependent[2–5] and a variety of other agents that modulate collagen metabolism inhibit angiogenesis,[7,10,11,14–18,24,28] it is possible that the cytostatic effects of minocycline and other tetracycline derivatives result, at least in part, from inhibition of tumor neovascularization.

Antiangiogenic Effect of Minocycline

In 1991, Tamargo, *et al*.,[25] showed that minocycline is a potent inhibitor of tumor angiogenesis in the rabbit cornea. The following year, Guerin and colleagues[9] reported that minocycline inhibited the growth of early-passage endothelial cells with an IC_{50} between 20 and 40 μM. At similar concentrations, minocycline had minimal effects on the growth of pericytes, smooth-muscle cells, and C6 glioma cells. Deoxyribonucleic acid and protein synthesis in endothelial cells, assessed by measuring the incorporation of tritiated thymidine and tritiated leucine *in vitro*, were also selectively inhibited by minocycline. Testing other tetracycline derivatives, the authors demonstrated that the potency of inhibition of endothelial cell growth correlated with relative anticollagenase effects, rather than with antimicrobial activity. Sotomayor, *et al*.,[23] demonstrated that minocycline has minimal cytotoxic effects against tumor cells *in vitro*. These observations lend further support to the hypothesis that the antitumor effect of minocycline is related to inhibition of angiogenesis.

Interstitial Drug Delivery Using Controlled-Release Polymers

Local delivery of drugs by controlled-release polymers has several advantages: 1) sustained drug delivery; 2) high local tissue concentrations without high systemic drug levels; and 3) bypass of the blood-brain barrier's restriction to drug delivery in the brain.[8,26,32] These drug delivery characteristics can aid delivery of antiangiogenic agents to brain tumors. First, effective long-term control of tumor growth with these agents depends on sustained delivery to the tumor, whether they work by inhibition of mitochondrial protein synthesis, by control of angiogenesis, or by both mechanisms. Second, to maximize the effect on the tumor and minimize systemic effects, high local concentrations with low systemic levels are desirable. Finally, the blood-brain barrier restricts the type of antiangiogenesis agents that might be evaluated. Local delivery systems, such as the polymer, bypass this barrier and thus do not restrict the type of agents that can be tested.

Conclusions

When tumors are treated with antiangiogenic agents, a balance exists between active vascularization of a rapidly growing tumor and inhibition of angiogenesis by the drug. If the net effect favors neovascularization, tumor growth will continue, albeit possibly at a slower rate. The timing of treatment and the vascular state of the tumor may be important determinants of clinical effectiveness of angiogenesis inhibitors. Indeed, we found that the ability of minocycline to prolong survival in rats with intracranial 9L glioma implants depends on the timing of treatment and the tumor state. If treatment with local minocycline began at the time of tumor implantation or after tumor resection, effective inhibition of tumor growth and prolonged survival resulted. If treatment was delayed for 5 days after implantation, minocycline did not increase survival time. However, if the state of the tumor was altered, either by BCNU or surgical resection, locally delivered minocycline significantly extended survival.

In summary, we have demonstrated that minocycline prolongs survival of rats with intracranial gliomas. The mechanism for the inhibition of tumor growth is not established, although it may be related to antiangiogenic activity, inhibition of mitochondrial protein synthesis in neoplastic cells, or both. Minocycline delivered locally by a controlled-release polymer is a clinically effective modulator of intracranial tumor growth, particularly when used in combination with a chemotherapeutic agent and surgical resection of the tumor.

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