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● *Biology Contribution*

SYNTHETIC, IMPLANTABLE POLYMERS FOR LOCAL DELIVERY OF IUDR TO EXPERIMENTAL HUMAN MALIGNANT GLIOMA

JEFFERY A. WILLIAMS, M.D.,*† XUAN YUAN, M.D.,*† LARRY E. DILLEHAY, PH.D.,*
VENKATRAM R. SHASTRI, PH.D.‡ HENRY BREM, M.D.,† AND JERRY R. WILLIAMS, SC.D.*

Radiobiology Laboratory, Division of Radiation Oncology, Department of Oncology, and †Brain Tumor Research Laboratory, Departments of Neurosurgery and Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD, ‡Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA

Purpose: Recently, polymeric controlled delivery of chemotherapy has been shown to improve survival of patients with malignant glioma. We evaluated whether we could similarly deliver halogenated pyrimidines to experimental intracranial human malignant glioma. To address this issue we studied the *in vitro* release from polymers and the *in vivo* drug delivery of IUdR to experimental human U251 glioblastoma xenografts.

Methods and Materials: *In vitro*: To measure release, increasing (10%, 30%, 50%) proportions of IUdR in synthetic [(poly(bis(p-carboxyphenoxy)-propane) (PCPP):sebacic acid (SA) polymer discs were serially incubated in buffered saline and the supernatant fractions were assayed. *In vivo*: To compare local versus systemic delivery, mice bearing flank xenografts had intratumoral or contralateral flank IUdR polymer (50% loading) treatments. Mice bearing intracranial (i.c.) xenografts had i.c. versus flank IUdR polymer treatments. Four or 8 days after implantation of polymers, mice were sacrificed and the percentage tumor cells that were labeled with IUdR was measured using quantitative microscopic immunohistochemistry.

Results: *In vitro*: Increasing percentage loadings of IUdR resulted in higher percentages of release: 43.7 + 0.1, 70.0 + 0.2, and 90.2 + 0.2 ($p < 0.001$ ANOVA) for the 10%, 30%, and 50% loadings, respectively. *In vivo*: For the flank tumors, both the ipsilateral and contralateral IUdR polymers resulted in similarly high percentages labeling of the tumors versus time. For the ipsilateral IUdR polymers, the percentage of tumor cellular labeling after 4 days versus 8 days was 45.8 ± 7.0 versus 40.6 ± 3.9 (p = NS). For the contralateral polymer implants, the percentage of tumor cellular labeling were 43.9 ± 10.1 versus 35.9 ± 5.2 (p = NS) measured 4 days versus 8 days after implantation. For the i.c. tumors treated with extracranial IUdR polymers, the percentage of tumor cellular labeling was low: 13.9 ± 8.8 and 11.2 ± 5.7 measured 4 and 8 days after implantation. For the i.c. tumors having the i.c. IUdR polymers, however, the percentage labeling was comparatively much higher: 34.3 ± 4.9 and 35.3 ± 4.0 on days 4 and 8, respectively. For the i.c. tumors, examination of the percentage cellular labeling versus distance from the implanted IUdR polymer showed that labeling was highest closest to the polymer disc.

Conclusion: Synthetic, implantable biodegradable polymers provide the local, controlled release of IUdR and result in the high, local delivery of IUdR to experimental intracranial human malignant glioma. This technique holds promise for the local delivery of IUdR for radiosensitization of human brain tumors. © **1998 Elsevier Science Inc.**

Biodegradable polymer, Iododeoxyuridine, Radiosensitization, Human glioma.

INTRODUCTION

For human malignant gliomas, halogenated pyrimidines are potent *in vitro* radiosensitizers (1, 2) and have shown clinical promise in the treatment of anaplastic astrocytoma (3). The extent of radiosensitization increases with the percentage of replacement of thymidine in replicating DNA (4–6) that in turn, correlates with both the concentration and duration of exposure to the halogenated pyrimidine. Although many clinical trials have been initiated, no study has shown improved survival following systemic administration of IUdR and radiotherapy (7–17). Several factors have limited the effectiveness of the clinical radiosensitization of malignant gliomas by IUdR. These limitations include rapid clearance of halogenated pyrimidines from the blood after systemic administration (11), significant toxicity (usually marrow suppression) (10, 16–19) and restriction of delivery to the brain by the blood-brain barrier (20). In clinical trials, the levels of halogenated pyrimidine incorporated into the cells of biopsied tumors are low and measure only approximately 4% (16, 17).

Polyanhydride polymers have been developed to deliver chemotherapeutic agents to brain tumors in a sustained,

Reprint requests to: Jeffery A. Williams, M.D., Harvey 811, The Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21205-8811. E-mail: jw@hu.edu

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controlled manner. The delivery system is FDA approved and has been shown to be safe and effective (21–23). Sustained, intracranial (i.c.) delivery of halopyrimidines could circumvent the blood-brain barrier, diminish systemic exposure, and offer potentially increased uptake in i.c. tumors. We therefore studied the release and resultant local delivery of IUdR to both i.c. and extracranial experimental human malignant glioma xenografts via synthetic, implantable polyanhydride polymers.

METHODS AND MATERIALS

Cells

The human malignant glioma cell line U251 was obtained from the DCT Tumor Repository, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. Cells were cultured in Dulbecco's MEM/Ham's F-12 nutrient mixture with 10% fetal bovine serum (GIBCO, Grand Island, NY) and antibiotics (penicillin and streptomycin) in 12 × 80 mm plastic culture dishes or T-25 culture flasks (Falcon Plastics, Cockeysville, MD). Cells were incubated at 37°C and gassed with a mixture of 5% carbon dioxide and 95% air. Medium was changed twice weekly and cells were passaged at confluence with 0.5% trypsin.

Polymer synthesis

The [(poly(bis(p-carboxyphenoxy)-propane) (PCPP):sebacic acid (SA) (PCPP:SA ratio 20:80) was synthesized according to the method of Domb and Langer (24) by Guilford Pharmaceuticals Inc., Baltimore, MD, who provided the polymer for these studies. IUdR (Sigma) and PCPP:SA were separately combined with methylene chloride (10% w/w). The two resultant solutions were combined to yield increasing (10%, 30%, or 50%) IUdR as a percentage of the total weight of the IUdR:polymer mixture. The solvent was evaporated under vacuum to yield a dry powder. Discs (10 mg final weight) were prepared by compression molding 11 mg of the powder with a stainless steel mold (internal diameter 2.5 mm) under light pressure using a vice.

In vitro IUdR release

Groups of at least three polymer discs having increasing (10%, 30%, or 50%) proportions of IUdR were incubated for known intervals in 1 ml 0.1 M phosphate-buffered saline (PBS) at 37°C. The supernatant fraction was periodically removed, replaced with fresh PBS, and assayed for IUdR by absorbance spectrophotometry. The cumulative percentages of the loaded IUdR that were released were plotted versus time. The plot of the released IUdR was fit to the exponential function of the form $y = a(t)^b$ where y is the cumulative released IUdR, a and b are constants, and t is time (h).

In vitro cellular labeling

Selective labeling of BUdR versus IUdR allowed simultaneous estimation of the BUdR labeling index versus IUdR labeling of cells following treatments. BR-3 and IU-4 monoclonal antibodies were purchased (Becton-Dickinson). The BR-3 reacts preferentially (50–100:1) with BUdR versus IUdR (25, 26). The IU-4 reacts equally with BUdR and IUdR. To measure the differential cellular labeling by the BR-3 (anti-BUdR) versus IU-4 (anti-IUdR and anti-BUdR) monoclonal antibodies, 10^6 U251 cells were incubated 0.5 h in media having 10 μM IUdR, 10 μM BUdR, combined 5 μM IUdR and 5 μM BUdR or combined 10 μM IUdR and 10 μM BUdR. After exposure, the cells were fixed (70% ethanol at −20°C) in the dark and prepared for fluorescence activated cell sorting (FACS) as described below.

Animals

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Male nu/nu nude mice, 6 weeks of age, were obtained from the National Cancer Institute (Bethesda, MD), kept in a dedicated animal facility with five mice/cage, and given free access to lab chow and water. Experimental groups included at least six mice. When indicated, mice were sacrificed via cervical dislocation.

Anesthesia

For surgical procedures, mice were anesthetized with i.p. injections (3 ml/kg) of a solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol in normal saline. The mice recovered in their cages following all surgical procedures.

Xenografts

Flank. U251 cells in log-phase growth were trypsinized, counted, and resuspended in culture medium. After preparation of the left flanks with 70% ethyl alcohol, mice had subcutaneous injections of 5×10^6 cells in a volume of 0.1 ml. Tumors were visible after 2 weeks for > 90% of animals. The tumors were approximately spherical in shape. To accommodate any deviation from symmetry in any or all axes, we used the formula for the volume of an ellipsoidal volume: $V = (II/6)(L \times W \times H)$. For the spheres, this formula reduces to $V = 4/3\ II(r^3)$. The size of experimental tumors was approximately 0.1 cm^3.

Flank tumor volumetrics. For flank tumors, measurements of the tumor length (L), width (W), and height (H) were made every 3 days. The product ($L \times W \times H$) is proportionate to tumor volume (V). The logarithm of the ratio of this product (V) to the initial product (V_0) (on day 0 when the experimental volume was reached) for each animal and for the average of each treatment group were measured versus time as previously described (27) and allowed measurements of tumor-doubling times.

Intracranial xenographs. U251 cells in log-phase growth were trypsinized, counted, and resuspended in culture medium in numbers to yield 2×10^5 cells per 10 μl. Anesthetized mice were placed in a stereotactic head frame (Kopf, Tujunga, CA). The coordinates for injection were 3 mm right of midline, 2 mm posterior to the coronal suture,

and 3 mm deep. The scalp was prepared with 70% ethyl alcohol. The 26-gauge syringe attached to the Hamilton syringe (50 μl) was advanced through the skin, skull, and brain to the target. After injection of 2×10^5 cells (10 μl) the syringe was left in place for 1 min before slow withdrawal.

Implantation of polymers

Flank. Using a #11 scalpel, single incisions were made over the tumors and contralateral flank. Groups of mice had concurrent implantations of 50% IUdR polymers to a depth of 3 mm in the tumor and empty (control) polymers in the subcutaneous contralateral flank. To test systemic delivery of the IUdR to the flank xenografts, mice had implantation of the empty polymer in the tumor and the IUdR polymer in the subcutaneous contralateral flank. After implantations of polymers, the skin edges were approximated using the single metal wound clips (Mikron Autoclip, Fisher Scientific).

Intracranial. On day 4 after the cellular inoculation, mice had midline sagittal incisions of the scalp using a #11 scalpel. Using a rotating burr, a craniectomy approximately 5 mm in diameter was created directly over the site of the prior cellular inoculation. Using forceps, single empty or IUdR polymers were inserted to a depth of 3 mm. To measure systemic delivery of IUdR to the i.c. tumors, mice bearing the brain xenografts had implantation of empty i.c. polymers and subcutaneous flank IUdR polymers. After implantations, the skin edges were approximated with a single metal wound clip.

Labeling index

Flank tumors and bone marrow. For calculations of the labeling indices for the flank xenografts, mice received 1 ml i.p. BUdR (30 mg/ml in Tris buffer [pH 9.0]) on day 4 or 8 after IUdR polymer treatments. After 6 h, the mice were sacrificed, the flank tumors were excised, bisected with the scalpel, and duplicate (mirror) sections approximately cuboidal in shape and weighing approximately 10 mg were taken from the center of the tumor. During excision, the tumors were readily discernible and separated from the surrounding muscle tissue. For harvesting of the marrow, both femurs and tibias were excised, trimmed of excess tissue using a scalpel, and the marrow purged by flushing 2 ml culture medium via a 25-gauge syringe. The tumor sections and marrow were separately fixed in 70% alcohol at 4°C and stored in the dark until preparation for fluorescence activated cell sorting as described below. One section per tumor was analyzed and its mirror section was saved for potential additional studies.

Preparation of tumor cellular nuclei

Within 2 weeks of sacrifice the flank tumor specimens and marrow were processed to yield nuclear suspensions. The i.c. tumors, however, were infiltrative, grossly indistinguishable from the surrounding normal brain, and were therefore not amenable to tumor cell isolation. The sections of flank tumors were isolated and minced into small fragments (about 1 mm in diameter) and digested 1 h in combined 0.1 N HCl and 0.4 mg/ml pepsin (Sigma) at 37°C using continuous agitation. The product was filtered through a 38-um nylon mesh and washed twice in PBS containing 1 mg/ml bovine serum albumin (Sigma) and 0.1 – 0.3% Tween 20 (Sigma). After centrifugation, the supernatant was discarded. To denature the DNA, 1 ml 2 N HCl was added to the pellet while vortexing. The solution was neutralized with 2 ml 0.1 M Borax buffer (Sigma) (pH 8.5) and washed twice in PBS. The pellet with the nuclei was resuspended in 200 ul of PBS, and 10 ul of BR-3 (mouse anti-bromodeoxyuridine) or IU-4 (anti-iododeoxyuridine) was added. The incubation proceeded for 1 h at 4°C in the dark. After washing twice in PBS, the pellet was resuspended in 200 μl PBS, and 3 μl of anti-mouse IgG fluorescein isothiocyanate (FITC)/conjugate (Sigma) was added and incubated for 1 h at 4°C (28–30). After washing twice in PBS, 1 ml of a solution containing 20 mg/ml PI (Sigma) and 5 μg/ml RNase (Sigma) was added and incubated for at least 30 min.

Flow cytometry

For flow cytometry of the nuclear preparations, emissions of FITC and PI were detected using 530 nm short pass and 630 nm long pass filters, respectively (31). Data were collected and bivariate displays of log FITC versus DNA content (PI) were created. A minimum of 10,000 events were analyzed per sample. The percentage of labeled cells was determined. The cutoff level for noise was readily discerned. The cell-cycle phase distribution was obtained from the DNA histogram analysis. Tumor growth reflects the competing processes of cellular production through division and cell loss. The potential doubling time (Tp) of a tumor reflects the higher rate of growth (shorter doubling time) of the tumor if there was no cell loss (32). The tumor potential doubling times (Tpot) were determined from the relative movement method as previously described (28, 33).

Immunohistochemistry

For the quantitation of the cellular labeling of both the i.c. and flank tumors by the IUdR, groups of mice bearing i.c. or flank tumors were sacrificed 4 or 8 days after implantation of the polymers. For removal of the tumors, the entire brain bearing the tumor or the flank tumor was immediately excised and fixed (10% formalin for 1 week). For preparation of tumors, the whole brain bearing the tumor or the s.c. flank tumor was bisected (coronally for brain; transaxially [across long axis if present] for flank tumors) at the site of polymer implantation using a #11 scalpel. Beginning from this cut surface, three sections of tissue, each 1-mm thick, were cut using a microtome. From each of the 1 mm sections, three sections, each 10-μm thick were cut, again beginning from the edge closer to the original cut surface of the specimen. These sections were processed for immunohistochemistry as follows. The tissue specimens were deparaffinized with xylene, rehydrated (sequential 100%, 95%, and 75% ethanol) and rinsed in distilled water. The

specimens were briefly digested using 200 μl pepsin (400 μg/ml) in 0.1 N HCl for 15 min at 37°C. Specimens were denatured with 4 N HCl for 10 min at room temperature, neutralized with 0.1 M Borax and triple-washed in PBS. Specimens were blocked with 3% BSA in PBS for 30 min and incubated with 1:10 solution of anti-IUdR in 0.5% BSA in PBS for 30 min at 37°C. Specimens were washed three times in PBS, and incubated with 1:1000 goat anti-mouse IgG (Amersham) in PBS for 45 min at room temperature. Specimens were washed and incubated with FITC-linked streptavidin (Amersham) for 30 min at room temperature. For DNA staining, specimens were washed with PBS and incubated for 15 min with 0.5 μg/ml propidium iodide (PI). Slides were washed with PBS and mounted in a 90%/10% glycerol/water medium containing 1 mg/ml p-phenylenediamine as an anti-fading agent.

For quantification of the cellular labeling by IUdR, three high-power fields (×400) each having at least 200 tumor cells were examined using the 530 nm (FITC) versus 630 nm (PI) filters. The numbers of labeled tumor cells were calculated as a percentage of the total tumor cells.

Statistical evaluation

For all studies the data are the mean + standard error of the mean (SEM). The comparisons of paired observations (*in vitro* labeling of IUdR vs. BUdR; percentage labeling of tumors for i.c. vs. flank polymer treatments) were compared by Student's *t*-test. The percentage labeling of tumors versus distance from polymers were compared by linear regression analysis. (34).

RESULTS

In vitro

IUdR release. The cumulative percentages of the IUdR that were released from the polymers are shown in Fig. 1. The early rates of release were high for all loadings of IUdR. The percentage of the loaded IUdR that was eventually released was proportionate to the percentage loading. After 1 day the average cumulative percentage of loaded IUdR released from the polymers having the lowest (10%) loading (34.7 ± 0.4) was less than that released from the polymers having the higher loadings: 51.4 ± 0.7 and 53.3 ± 0.7 ($p < 0.001$ ANOVA vs. 10% loading) for the 30% and 50% loadings, respectively. After 4 days the cumulative percentages were 43.7% ± 0.1, 70.0% ± 0.2, and 90.2% ± 0.2 ($p = < 0.001$ ANOVA) for the 10%, 30%, and 50% loadings. The percentage IUdR released versus time was fit to the exponential function of the form $y = a(t)^b$ where y is the cumulative released IUdR, a and b are constants, and t is time (h). Values for a, b (R^2) (correlation coefficient) were 32.3, 0.24 (0.94) ($p < 0.00001$ ANOVA) for 10%; 43.0, 0.33 (0.92) ($p = 0.0003$) for 30%; and 44.6, 0.45 (0.94) ($p = 0.0002$) for 50% loadings of IUdR. These results confirmed and quantified the controlled release and provided the basis for the subsequent quantification of the delivery of the IUdR to the experimental tumors.

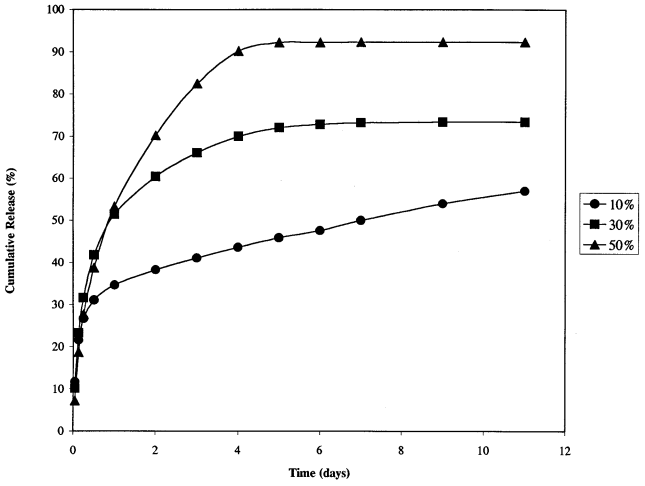

Fig. 1. Release of IUdR from polymers. Individual polymers were incubated (0.1 M PBS; 37°C), and the supernatant was serially removed and assayed for IUdR. Ordinate: Percentage of loaded IUdR released into medium. Abscissa: Time (Days). Symbols: 10%, 30%, 50% loading. The SEM was less than 1% for all points.

Fluorescence activated cell sorting

In vitro cellular labeling. The percentages of the U251 cells that were labeled by the IU-4 antibody after the 10 μM IUdR alone, 10 μM BUdR alone, and the combined incubations (5 μM IUdR and 5 μM BUdR or 10 μM IUdR and 10 μM BUdR, were similar (Table 1). These results showed that the selectivity of the IU-4 antibody for both the IUdR and the BUdR is similar.

The percentages of cells labeled by the BR-3 antibody after the same treatments, however, were very low after the IUdR incubation, highest for the BUdR incubation, and intermediate for the combined incubations (Table 1). These results showed the specificity of the BR-3 antibody for the BUdR. Further, these results provided the basis for the *in vivo* studies of double labeling via i.p. BUdR versus polymer-mediated delivery of IUdR as described below. These results further provided the experimental basis for the calculations of both the duration of the DNA synthesis phase (Ts) and the potential doubling time (Tpot) for the flank tumors.

In vivo cellular labeling. As the tumors grew larger the labeling index decreased. The BUdR labeling indices of

Table 1. Cellular labeling: IUdR versus BUdR *in vitro*

Addition to medium	Antibody probe IU-4 % cells labeled	BR-3 % cells labeled
10 uM IUdR	52 ± 1	2 ± 1**
10 uM BUdR	47 ± 2	55 ± 2
Combined (5 uM IUdR + 5 uM BUdR)	53 ± 1	32 ± 2*
Combined (10 uM IUdR + 10 uM BUdR)	53 ± 2	44 ± 2

* $p < 0.05$.
** $p < 0.01$.

Table 2. Comparison of tumor cell labeling: i.p. BUdR and/or IUdR polymer

Labeling treatments		Excision	Antibody probe		
			IU-4	BR-3	
BUdR	IUdR	(day)	% cells labeled		$p^{\dagger}$
i.p.	None	4	7 ± 1	7 ± 1	NS
		8	4 ± 1	4 ± 1	NS
None	Tumor polymer	4	47 ± 1	1 ± 0**	< 0.01
		8	45 ± 7	1 ± 1**	< 0.01
i.p.	Contralateral polymer	4	67 ± 2	11 ± 1**	< 0.01
		8	51 ± 3	8 ± 1**	< 0.01
i.p.	Tumor polymer	4	74 ± 3	13 ± 3**	< 0.01
		8	66 ± 8	9 ± 2**	< 0.01

* $p < 0.05$.
** $p < 0.01$.
† p: Comparisons of percentage cellular labeling.

7.4 ± 1.0 versus 3.8 ± 1.4% were recorded 4 days versus 8 days after reaching the experimental volume (0.1 cc) (Table 2). The percentages of cellular labeling were very high both 4 and 8 days after the IUdR polymer treatments (Table 2). The BR-3 antibody that is specific for BUdR, however, reported very low percentages of cellular labeling after the IUdR polymer treatments. These results provided the experimental basis for the combined (double) labeling of the tumors by the IUdR (delivered by the polymers) and by the BUdR (delivered by the i.p. treatments). After the IUdR polymer implantation contralateral to the tumor and i.p. BUdR treatments, the mean percentage of labeling for the BR-3 and IU-4 antibodies was 10.6% + 2.0 and 66.7% + 2.4 ($p < 0.001$), respectively. When corrected for the binding of IUdR by the BR-3, these results are consistent with the results of the i.p. BUdR treatments alone that are described above. For the IUdR polymer treatment ipsilateral to the tumor and subsequent i.p. BUdR, the percentage labeling for the BR-3 and IU-4 antibodies was 13.4% + 3.3 and 74.0% + 3.3 ($p < 0.001$) respectively. The difference between the tumor IUdR labeling after the ipsilateral versus contralateral IUdR polymer treatments was not significant.

Tumor doubling times. In the first 4 days after IUdR polymer implant the volume doubling time (days) was 4.4 + 0.4 (Fig. 2). The potential doubling times (Tpot) measured by the relative movement method (18, 28) were not different (4.5 ± 0.6 vs. 4.5 ± 0.4 days [p = NS]) for tumors measured on day 4 versus day 8 after reaching the size for treatments. The close approximations of the actual doubling times and the potential doubling times may indicate that the cell loss factor is small in this system, consistent with the observations of Perez (35). In that study, the U251 cell line showed the smallest cell loss factor of 10 studied human tumor xenografts. Further, the durations (h) of DNA synthesis (Ts) were also similar (9.8 ± 0.4 vs. 8.9 ± 0.2 [p = NS]) for the tumors harvested on day 4 versus day 8.

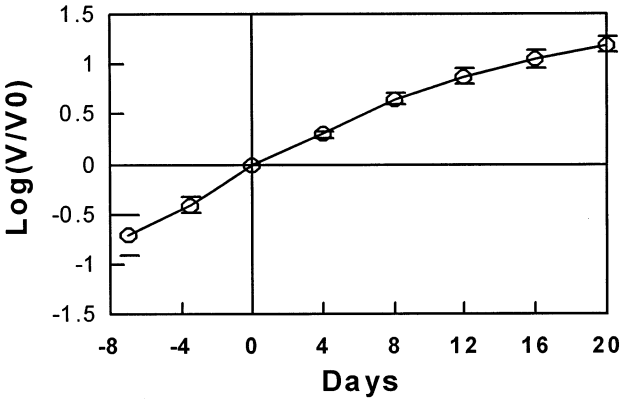

Fig. 2. Tumor growth. For measurement of flank tumor growth, the tumor length (L), width (W), and height (H) were recorded every 3 days. The formula for tumor volumes was V = (II/6)(L × W × H). Day 0 was the date of achievement of experimental tumor volume (0.1 cc) tumor volume. The product (L × W × H) is proportionate to tumor volume (V). The logarithm of the ratio of this product (V) to the initial product (V_0) (on Day 0 when the experimental volume was reached) for each animal was measured versus time as previously described (28). The mean log V/V0 is plotted versus time. Error bars: SEM.

Immunohistochemical cellular labeling/flank. Control (empty) polymer treatments resulted in no observed fluorescence of the FITC in the histological samples. For the tumors having ipsilateral IUdR polymer treatments, the percentage of tumor cellular labeling on day 4 versus day 8 after implantation were similar (Table 3). For IUdR tumor polymer the percentage of labeling decreased with distance. Linear regression analysis showed that this decrease versus distance was significant. For the IUdR polymer treatments contralateral to the tumor, the percentage of tumor cellular labeling was very similar to that following the ipsilateral treatments. When the ipsilateral versus contralateral treatments were compared for each interval (0, 1, or 2 mm), the observed differences in the percentage of cellular labeling was not significant.

Brain xenografts

Immunofluorescent cellular labeling. Control (empty) polymer treatments resulted in no observed labeling of the tumor cells. For the flank IUdR polymer treatments of the brain tumors, the percentage of cellular labeling was comparatively low (Table 4). For the i.c. IUdR polymer treatments of the brain tumors, however, the percentage of cellular labeling was comparatively higher. When the percentage of cellular labeling was compared for the i.c. versus flank polymer treatments, the difference was significant for all of the day 8 measurements, but not for the day 4 measurements.

IudR labeling of bone marrow. After the flank or i.c. IUdR polymer treatments, labeling of the bone marrow was observed (Table 5). No significant temporal pattern of labeling was observed for the 4 day versus 8 day measurements for the flank versus i.c. treatments, respectively.

Table 3. Tumor cellular labeling versus distance from polymers

IUdR Polymer location	Excision (day)	Distance from tumor polymer 0 mm	1 mm % cells labeled	2 mm	Animals/ group	$p^{\dagger}$
Tumor	4*	46 ± 7	25 ± 9	18 ± 9	6	0.012*
	8**	41 ± 4	32 ± 5	25 ± 4	5	0.004**
Contralateral	4	44 ± 10	31 ± 10	31 ± 8	6	0.90
	8	36 ± 5	36 ± 8	32 ± 7	6	0.63

* $p < 0.05$.
** $p < 0.01$.
† p: Linear regression analysis: percentage cellular labeling versus distance from polymers. Significance of non-zero slope of measured values versus distance.

DISCUSSION

In vitro

IUdR polymer release kinetics. To label and sensitize tumor cells *in vivo*, the controlled, protracted administration of IUdR is particularly important. For substitution of thymidine and resultant radiosensitization of tumors, prolonged exposure to IUdR is particularly desirable. For the *in vitro* polymers, the initial rates of release of the IUdR were very high for each of the percentage loadings of the IUdR. After the initial high rate, the eventual rate of release of IUdR was both slower and protracted over a duration of approximately 5 days. A high percentage of substitution requires exposure during multiple cellular divisions; therefore, the duration of the release of IUdR is critical for the potential radiosensitization of brain tumors.

A single monoexponential function characterized the continuous rates of release for each percentage loading of IUdR in polymers. The characterization of the sustained release by a single simple expression is confirmation of the controlled nature of the release. The polyanhydride polymers undergo hydrolytic bond cleavage to form water-soluble degradation products (36, 37). This polyanhydride polymer is attractive because the ratios of the two monomers (para-carboxyphenoxypropane and sebacic acid) in the polymer allow control over the rate of degradation. Thus, increasing the percentage of the hydrophilic monomer (sebacic acid) in the polymer increases the rate of entry of water into the polymer, resulting in faster release of the loaded drug. For example, the rate of erosion of the polymer ($\mu g/cm^2/h$ was 6.0, 80.0, and 160.0 for the PCPP:SA ratios of 85:15, 45:55, and 21:79, respectively (36). The IUdR is water-soluble (38). The larger percentage loadings of the IUdR may thus result in the increased rate of entry of water into the polymer, increased lysis of the anhydride bonds joining the monomers, and resultant higher release of the loaded drug.

The duration of the release of IUdR is compatible with the time that is required for thymidine replacement in replicating cells. Lawrence *et al.* (6) have shown that the incorporation of halogenated pyrimidines plateaued after 4 days of exposure to either IUdR or BUdR. For *in vivo* applications, however, the potential doubling times for tumors are comparatively more prolonged than for *in vitro* applications. Therefore, comparatively more protracted exposures may be necessary to achieve the maximal replacement of thymidine for radiosensitization *in vivo*.

Table 4. IUdR labeling of intracranial tumors: effect of distance and IUdR polymer location

Polymer location	Excision (day)	Distance from tumor polymer 0 mm	1 mm % cells labeled	2 mm	Animals/ group	$p^{\dagger}$
Brain	4	34 ± 5	18 ± 5	10 ± 2	6	0.003**
	8	35 ± 4	16 ± 3	10 ± 3	5	0.001**
Flank	4	14 ± 9	10 ± 7	7 ± 6	5	0.83
	8	11 ± 3	7 ± 1	2 ± 1	5	0.02
		$\rho^{\ddagger}$: Brain versus flank IUdR polymers				
	Day 4	0.07	0.35	0.70		
	Day 8	0.001**	0.02*	0.04*		

* $p < 0.05$.
** $p < 0.01$.
$p^{\dagger}$: Linear regression analysis: The percentage cellular labeling is analyzed verus distance from polymers. The significance of the non-zero slope of percentage labeling versus distance is shown.
$p^{\ddagger}$: Comparison of paired observations: Percentage cellular labeling for intracranial versus flank IUdR polymer treatments for each distance from the polymer.

Table 5. Labeling of bone marrow cells

IUdR polymer treatment	IUdR labeling (%)	
	Day 4	Day 8
Brain polymer	49.1 ± 8.9	63.1 ± 11.2
Flank polymer	66.3 ± 11.2	39.6 ± 2.7*

* $p < 0.05$ versus day 4.

In vivo

Flank xenografts. The labeling of cells by IUdR was very high both 4 and 8 days after the IUdR polymer treatments. The described labeling indices, duration of the S phase, and potential doubling times for the xenografts are consistent with those of other experimental xenograft systems (1). Further, these results are similar to labeling studies after the intravenous administrations of halogenated pyrimidines. In studies of the continuous systemic infusion of BUdR in mice bearing D-54 human malignant glioma xenografts, thymidine replacement by BUdR reached equilibrium after 7 days (1). Thus, the observed duration of release of IUdR by the experimental PCPP:SA polymers in the current study could coincide with the required interval for the high replacement of thymidine in the experimental human malignant glioma xenografts. Further, that study (1) found higher rates of thymidine replacement by BUdR after shorter infusion schedules of higher amounts (400 mg/kg/day for 5 days) versus 100 mg/kg/day for 14 days (10.3 + 0.4 vs. 6.0 + 0.6% replacement of thymidine). For these studies the rates of thymidine replacement in the marrow was high, measuring approximately three-fold higher than the replacement in the tumor. Further, the highest infusion rate showed high toxicity for the animals and was only tolerated for 5 days. Using polymer delivery, we were able to use approximately one half of this dose of halogenated pyrimidine to result in a very high percentage of cellular labeling.

The current results also compare favorably with the studies of intravenous administrations of halogenated pyrimidines to experimental colon carcinoma flank xenografts (39). In that study, mice received continuous i.v. administration of IUdR at a rate of 100 mg/kg/day for 5 days. Assuming the average weight of each mouse was 25 g, mice therefore received 12.5 mg total IUdR that resulted in labeling 94% of the cells from the colon carcinoma xenografts. We have shown that with polymer treatments, each mouse received 5 mg IUdR that resulted in the labeling of 47% of the tumor cells. For these polymer treatments, a larger animal model may facilitate larger polymer discs or multiple implantations of polymer discs to yield higher molar IUdR administrations with potentially higher resultant cellular labeling.

The similar observed percentage of cellular labeling for the tumors having ipsilateral versus contralateral polymer treatments shows that for the flank tumor model, the local placement of the polymer did not appear to confer any advantage over distant implantation in terms of the labeling of the tumor cells. These results are very consistent with our prior study of the radiosensitization of the flank xenografts by the IUdR polymers (40). In that study, the rates of growth delay for the U251 flank xenografts after external beam irradiation were similar for both the ipsilateral and contralateral IUdR polymer treatments, suggesting equivalent delivery from the two distinct sites of implantation.

Marrow. The rates of marrow labeling were very high for the flank polymer treatments and are consistent with delivery of IUdR via the bloodstream. The rates of labeling were higher initially (day 4 after IUdR polymer implantation) versus later (day 8). This result may be due to higher initial release of the IUdR by the polymers as reflected by the *in vitro* studies. Further, the equivalent labeling of tumor and marrow by the IUdR may represent an improved ratio when compared to the intensive intravenous administration of the halogenated pyrimidine (1).

Intracranial xenografts

Immunohistochemistry. The results show high and prolonged labeling of the i.c. xenografts with the IUdR both 4 and 8 days after the i.c. polymer treatments. In contrast to other studies of delivery of the halogenated pyrimidines to flank xenografts, no studies exist for the comparison of the methods for the delivery of IUdR to the i.c. xenografts. This comparison may be important in view of the critical factors that have combined to render the current peripheral administrations of IUdR ineffective for the achievement of radiosensitization of brain tumors. These factors include both the blood-brain barrier (16, 41, 42) and the high peripheral toxicity (marrow) that is dose-limiting after systemic administration of the halogenated pyrimidines.

The percentage of labeled i.c. tumor cells was similar to the percentage labeling of the flank xenografts. For the i.c. treatments, the labeling was highest for the tumor sections closest to the polymer. This result suggests that local diffusion, rather than perfusion by the i.c. vascular system, results in a gradient of concentration of IUdR that is highest closest to the polymer.

The persistence of high labeling 8 days versus 4 days after polymer implantation suggests local retention of the IUdR after release from the polymer. The studies of release showed that approximately 90% of the IUdR was released after 5 days. The high i.c. tumor cellular labeling 8 days after the polymer implantation suggests retention of the IUdR in the i.c. space after release from the polymer, that may result in prolonged exposure, and hence, cellular labeling.

Flank polymer/brain tumor. The diminished labeling of the i.c. tumor by the flank IUdR polymer treatment suggests the potential importance of both the blood-brain barrier and the known high rates of peripheral degradation combining to preclude high delivery of IUdR. These results demon-

strate the importance of the local delivery of IUdR to the i.c. tumor xenografts.

Marrow. Following the i.c. IUdR polymers, the initial (day 4 after polymer implantation) percentage of labeling of the marrow was lower than for the later (day 8) measurement. Thus, the temporal pattern of labeling is reversed when compared to that following the flank polymer treatments. This reversal of the temporal pattern may be due to a relative delay in the egress of IUdR from the i.c. compartment and the subsequent exposure of the marrow.

CONCLUSION

Implantable, biodegradable polymers offer the controlled release of IUdR that in turn results in the high labeling of both i.c. and flank experimental human glioblastoma xenografts. These results suggest that intraoperative placement of IUdR polymers during resection of human brain tumors may result in significant labeling of the tumor that may allow successful postoperative radiosensitization.

REFERENCES

1. McLaughlin P, Lawrence T, Seabury H, *et al.* Bromodeoxyuridine-mediated radiosensitization in human glioma: The effect of concentration, duration, and fluoropyrimidine modulation. *Int J Radiat Oncol Biol Phys* 1994; 30:601–607.
2. Uhl V, Phillips T, Ross G, *et al.* Iododeoxyuridine incorporation and radiosensitization in three human tumor cell lines. *Int J Radiat Oncol Biol Phys* 1992; 22:489–494.
3. Levin V, Prados M, Wara W, *et al.* Radiation therapy and bromodeoxyuridine chemotherapy followed by procarbazine, lomustine, and vincristine for the treatment of anaplastic gliomas. *Int J Radiat Oncol Biol Phys* 1995; 32:75–83.
4. Erickson R, Szybalski W. Molecular radiobiology of human cell lines. V. Comparative radiosensitizing properties of 5-halodeoxycytidines and 5-halodeoxyuridines. *Radiat Res* 1963; 20:252–262.
5. Fornace A, Dobson P, Kinsella T. Enhancement of radiation damage in cellular DNA following unifilar substitution with iododeoxyuridine. *Int J Radiat Oncol Biol Phys* 1990; 18:873–878.
6. Lawrence T, Davis M, Maybaum J, *et al.* The dependence of halogenated pyrimidine incorporation and radiosensitization on the duration of drug exposure. *Int J Radiat Oncol Biol Phys* 1990; 18:1393–1398.
7. Goffinet D, Brown J. Comparison of intravenous and intra-arterial pyrimidine infusion as a means of radiosensitizing tumors. *Radiology* 1977; 124:819–822.
8. Russo A, Gianni L, Kinsella T, *et al.* Pharmacological evaluation of intravenous delivery of 5-bromodeoxyuridine to patients with brain tumors. *Cancer Res* 1984; 44:702–1705
9. Jackson D, Kinsella T, Rowland J, *et al.* Halogenated pyrimidines as radiosensitizers in the treatment of glioblastoma multiforme. *Am J Clin Oncol* 1987; 10:437–443.
10. Kinsella T, Russo A, Mitchell J, *et al.* Phase I study of intravenous iododeoxyuridine as a clinical radiosensitizer. *Int J Radiat Oncol Biol Phys* 1985; 11:1941–1946.
11. Kinsella T, Collins J, Rowlan J, *et al.* Pharmacology and phase I/II study of continuous intravenous infusions of iododeoxyuridine and hyperfractionated radiotherapy in patients with glioblastoma multiforme. *J Clin Oncol* 1988; 6:871–879.
12. Greenberg H, Chandler W, Ensminger W. Radiosensitization with carotid arterial infusion bromodeoxyuridine and external beam radiation for gliomas. In: Ensminger W, Selam J, editors. Update in drug delivery systems. Mount Kisco: Futura Publishing Co.; 1989. pp. 233–246.
13. Hegarty T, Thornton A, Diaz R, *et al.* Intra-arterial bromodeoxyuridine radiosensitization of malignant gliomas. *Int J Radiat Oncol Biol Phys* 1990; 19:421–428.
14. Wirtanen G, Wiley A, Vermund H, *et al.* Intraarterial iododeoxyuridine infusion combined with irradiation. A pilot study. *Am J Clin Oncol* 1990; 13:320–323.
15. Cook J, Glass J, Lebovics R, *et al.* Measurement of thymidine replacement in patients with high grade gliomas, head and neck tumors, and high grade sarcomas after continuous intravenous infusion of 5-iododeoxyuridine. *Cancer Res* 1992; 52:719–725.
16. Goffman T, Dachowsk L, Bobo H, *et al.* Long-term follow-up on national cancer institute phase I/II study of glioblastoma multiforme treated with iododeoxyuridine and hyperfractionated irradiation. *J Clin Oncol* 1992; 10:264–268.
17. Urtasun R, Cosmatos D, DelRowe J, *et al.* Iododeoxyuridine (IUdR) combined with radiation in the treatment of malignant glioma: A comparison of short versus long intravenous dose schedules (RTOG 86-12). *Int J Radiat Oncol Biol Phys* 1993; 27:207–214.
18. Bagshaw MA, Doggett RS, Smith KC, *et al.* Intra-arterial 5-bromodeoxyuridine and x-ray therapy. *Am J Roentgenol* 1967; 4:886–894.
19. Phuphanich S, Levin EM, Levin VA. Phase I study of intravenous bromodeoxyuridine used concomitantly with radiation therapy in patients with primary malignant brain tumors. *Int J Radiat Oncol Biol Phys* 1984; 10:1769–1772.
20. Pollay M, Roberts P. Blood-brain barrier: A definition of normal and altered function. *Neurosurgery* 1980; 6:675–685.
21. Brem H. Polymers to treat brain tumors. *Biomaterials* 1990; 11:699–701.
22. Brem H, Piantadosi S, Burger P, *et al.* Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. *Lancet* 1995; 345:1008–1012.
23. Walter K, Cahan M, Gur A, *et al.* Interstitial taxol delivered from a biodegradable polymer implant against experimental malignant glioma. *Cancer Res* 1994; 54:2207–2212.
24. Domb A, Langer R. Polyanhydrides. I. Preparation of high molecular weight polyanhydrides. *J Polymer Sci* 1987; 25: 3373–3386.
25. Gratzner H. Monoclonal antibody to 5-bromo-and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 1982; 218:474–475.
26. Vanderlaan M, Thomas C. Characterization of monoclonal antibodies to bromodeoxyuridine. *Cytometry* 1985; 6:501–505.
27. Williams J, Klein J, Wanek P, *et al.* Quantitative intercomparison of radiolabeled antibodies and external beam radiotherapy in the treatment of human glioma xenografts *in vivo*. *Int J Radiat Oncol Biol Phys* 1992; 24:111–117.
28. Ritter M, Fowler J, Kim Y, *et al.* Single biopsy tumor kinetic analysis: A comparison of methods and an extension to shorter sampling intervals. *Int J Radiat Oncol Biol Phys.* 1992; 23: 811–820.
29. Schutte B, Reynders M, Assche C. An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry. *Cytometry* 1987; 8:372–378.
30. White R, Terry N. A quantitative method for evaluating biva-

riate flow cytometric data obtained using monoclonal antibodies to bromodeoxyuridine. *Cytometry* 1992; 13:490–495.
31. Fowler J, Lindstrom M. Loss of local control with prolongation in radiotherapy. *Int J Radiat Oncol Biol Phys* 1992; 23:457–467.
32. Hall EJ. Radiobiology for the radiologist. Philadelphia: Lippincott; 1994. p. 201.
33. Begg A, McNally N, Schrieve D, *et al.* A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* 1985; 6:620–626.
34. Norusis M. SPSS Advanced Statistics 6.1. Chicago: SPSS Inc.; 1994.
35. Perez LA, Dombkowski D, Efird J, *et al.* Cell proliferation kinetics in human tumor xenografts measured with iododeoxyuridine labeling and flow cytometry: A study of heterogeneity and a comparison between different methods of calculation and other proliferation measurements. *Cancer Res* 1995; 55:392–398.
36. Leong KW, Brott BC, Langer R. Bioerodible polyanhydrides as drug-carrier matrices. I: Characterization, degradation, and release characteristics. *J Biomed Mater Res* 1985; 19:941–955.
37. Tamada JA, Langer R. Erosion kinetics of hydrolytically degradable polymers. *Proc Natl Acad Sci USA* 1993; 90:552–556.
38. Budavari S, ed. The Merck Index. Whitehouse Station: Merck; 1996. p. 841.
39. Rodriguez R, Ritter M, Fowler J, *et al.* Kinetics of cell labeling and thymidine replacement after continuous infusion of halogenated pyrimidines *in vivo*. *Int J Radiat Oncol Biol Phys* 1994; 29:105–113.
40. Williams J, Dillehay L, Sipos E, *et al.* Implantable biodegradable polymers for radiosensitization of human glioma *in vivo*. *J Neurooncol* 1997; 32:181–192.
41. Ghosh MK, Mitra AK. Enhanced delivery of 5-iodo-2′-deoxyuridne to the brain parenchyma. *Pharm Res* 1992; 9:1173–1176.
42. Ghosh MK, Mitra AK. Brain parenchymal metabolism of 5-iodo-2′-deoxyuridine and 5′-ester prodrugs. *Pharm Res* 1992; 9:1048–1052.