

Tumor Radiosensitization by Sustained Intratumoral Release of Bromodeoxyuridine

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ABSTRACT

We have previously reported that the use of the polymer bis(p-carboxyphenoxy)propane-sebacic acid (20:80) for intratumoral delivery of cisplatin in a mouse tumor model (RIF-1) potentiated the effects of acute and fractionated radiation. This mode of drug delivery seems particularly applicable to the administration of radiosensitizing drugs because an optimum concentration of radiosensitizer can be maintained in the tumor over the prolonged period required for fractionated radiation treatment. We have now investigated, in the same tumor model, radiosensitization by the thymidine analogue bromodeoxyuridine (BrdUrd).

BrdUrd (20%, w/w) was incorporated into bis(p-carboxyphenoxy)propane-sebacic acid (20:80) and polymer rods containing the drug implanted in the RIF-1 tumor. Preliminary *in vitro* studies of the rate of release of BrdUrd from the polymer showed an initial rapid loss over 24 h, followed by a slower release extending over the next 5 days. In experiments in which tumor cells, which had incorporated BrdUrd *in vivo* from implanted polymer, were excised and a single cell suspension irradiated *in vitro* radiosensitization indicative of BrdUrd incorporation was associated mainly with an increase in the α constant for the linear quadratic model of cell survival. Radiosensitization was seen for tumor cells harvested between 5 and 10 days after polymer implant, a finding that is consistent with results of experiments in which the percentage of cells that had incorporated BrdUrd were measured by flow cytometry at various times after polymer/BrdUrd implant. The proportion of tumor cells positive for BrdUrd was 40–50% between 3 and 8 days after polymer implant.

When tumors were irradiated *in situ* and response measured in terms of tumor growth delay (TGD), radiosensitization was not seen for an acute dose of 16.5 Gy. In contrast, significant radiosensitization was seen for fractionated treatments when polymer/BrdUrd was implanted 3 days before the first radiation dose. For a dose of 5×6 Gy, TGD was increased from 22 days for radiation alone to 27 days for radiation plus polymer implant. For 10×6 Gy fractions, TGD increased from 45–77 days for those mice in whom the tumor eventually regrew, whereas for 25% of the mice in this group the tumor volume was reduced to a point where it was no longer detectable and there was no recurrence for at least 120 days after treatment.

INTRODUCTION

Attempts to improve the response of patients to radiation treatment have involved a number of stratagems, including the use of radiosensitizing drugs. The finding that halogenated pyrimidine analogues (BrdUrd² and IdUrd), which compete with thymidine for incorporation into DNA, sensitize cultured cells and implanted tumors to ionizing and UV radiation (1) has led to the development of clinical protocols using these agents. Thymidine substitution by halogenated analogues radiosensitizes by an enhancement of the yield of radiation-induced double-strand breaks and other forms of DNA damage (2). A direct correlation between the increase in radiosensitivity and the level of thymidine substitution has been established in several malignant cell lines of rodent and human origin (3–5).

Results of clinical trials combining halogenated pyrimidines with fractionated radiation have had mixed results. In one study, patients with glioblastoma multiforme showed a dose-dependent response, with those patients who tolerated large, cumulative doses of BrdUrd having improved progression-free survival (6). In contrast, a retrospective analysis of more than 2000 patients with malignant glioma could not provide a definitive answer as to whether BrdUrd, given during radiotherapy, improved survival (7). Treatment of disease other than glioma has been reported to be more successful. For primary and metastatic sarcomas, control of local disease was reported to be good (8), whereas for head and neck tumors, which showed a high degree of IdUrd labeling, tumor response was excellent, with a number of complete remissions (9).

Useful radiosensitization with thymidine analogues depends both on having a sufficient uptake of the analogue into DNA on a per cell basis and on maximizing the proportion of cells with thymidine replacement (3, 10). Optimization of thymidine analogue incorporation has yet to be achieved in a clinical setting because the prolonged infusion of these drugs at a dose level that would produce maximum sensitization is likely to be accompanied by an unacceptable level of systemic toxicity (11). A sustained release intratumoral drug delivery system, such as a biodegradable polymer implant, would localize the drug to the tumor and release it slowly over time (12). In a typical device of this kind, the drug is trapped physically within the polymer matrix and is released as the polymer degrades in response to its local environment (12). The degradation rate can be adjusted by using different polymers or combinations of copolymers, and the implant degrades completely by the end of treatment.

A biodegradable copolymer implant of CPP:SA (20:80), initially developed by Leong *et al.* (13), which has good drug release characteristics and benign degradation products (14), has been tested in a number of experimental studies. Using a rat model of malignant glioma drug delivery by intracranial implant of the CPP:SA copolymer combined with Taxol (15), with buthionine sulfoximine to potentiate the effects of 4-hydroperoxycyclophosphamide (16) and with BCNU (17), have been reported. In all cases, rats treated with implanted polymer/drug showed improved survival compared with untreated animals or with those treated by systemic drug administration. Williams *et al.* (18) reported that implantation of 50% IdUrd polymers, using the CPP:SA copolymer, in a xenograft model of a human glioma was more effective for tumor control than radiation alone. At the clinical level, Phase I-III trials have demonstrated that 3.8% w/w BCNU incorporated into CPP:SA is safe and effective for treatment of patients with recurrent malignant glioma (19, 20).

We have reported the use of the same polymer, CPP:SA (20:80), for intratumoral delivery of cisplatin in a mouse tumor model (RIF-1). The concentration of drug in the tumor was enhanced, and tumor growth was significantly delayed when drug delivery was by cisplatin/polymer implants compared with systemic administration (21, 22). Cisplatin/polymer implant potentiated the effects of acute and fractionated radiation (23). In this study, we describe experiments to determine the effectiveness of radiosensitization by BrdUrd when the drug is delivered by intratumoral polymer implant. Results obtained in the preclinical model suggest that this stratagem might be a means to realize the clinical potential of radiosensitization by halogenated pyrimidines.

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² The abbreviations used are: BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; CPP, 1,2-bis(p-carboxyphenoxy)propane; CPP:SA, bis(p-carboxyphenoxy)propane-sebacic acid; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; TGD, tumor growth delay.

MATERIALS AND METHODS

Radiation. The tumors were irradiated with ^{60}Co gamma rays using a Theratron 780 unit at a dose rate of ~ 1.0 Gy/min. The mice were anesthetized, and the tumor only was irradiated while the rest of the body was shielded with 5-cm thick lead blocks. The dose levels to the tumor and other parts of the body were measured using thermoluminescent (TLD-100) LiF crystal dosimeters. The dosimeters were calibrated with the ^{60}Co beam, and their accuracy was found to be within 2–3%. The dose to the shielded portion of the body was $<4\%$ of the tumor dose.

Polymer Implant. The CPP:SA polymer was synthesized according to the procedure published in the literature (13). Briefly, CPP was first synthesized from p-hydroxybenzoic acid and 1,3-dibromopropane in a basic aqueous medium. The prepolymers of sebacic acid and CPP were then prepared by reaction with acetic anhydride. The final polymer product was prepared from a mixture of the prepolymers (molar ratio, 80:20, SA:CPP) in a polycondensation reaction carried out at ~ 200 – 220°C *in vacuo*. The crude product was purified by precipitation ($\text{CHCl}_3/\text{pet. ether}$) and finally washed with diethyl ether before being dried under vacuum. The polymer and BrdUrd (20% w/w; Sigma Chemical Co.) were ground together to form a fine, homogeneous powder that was subsequently heated to 80°C and extruded through an Eppendorf Combitip (tip diameter, 0.5 mm). The resulting rods were cooled at room temperature and stored in a desiccator until required.

In Vitro Degradation. Polymer rods containing BrdUrd, prepared as described, were placed in PBS at 37°C . The solutions containing released drug and polymer product were collected daily, and fresh PBS was added to the container. The amount of BrdUrd released into the medium was measured spectrophotometrically. Control experiments were done using polymer rods without incorporated BrdUrd.

Tissue Culture. The procedures for maintaining the RIF-1 cell line and growing tumors have been published previously (22). Briefly, cells were passaged using standard tissue culture techniques in α modification of MEM supplemented with 10% fetal bovine serum and 1% antibiotic-mycotic solution (all supplied by Life Technologies, Inc.). Tumors were initiated by s.c. injection of 2×10^5 cells into the backs of C3H mice (female, 6 weeks of age, 20 g; Charles River). Tumors appeared within 10 days and reached a volume of 94–130 mm^3 within 3 weeks. Tumor volumes were calculated from measurements taken at three orthogonal angles using the formula ($a*b*c*\pi/6$).

Treatments. Treatments were begun when the tumors reached a volume of ~ 100 mm^3 . Tumor-bearing mice were separated into treatment groups of six to eight mice. For implant of polymer rods, the mice were anesthetized (Nembutal, 65 mg/kg), the skin punctured with a (20-gauge) hypodermic needle and the polymer rod was inserted into the tumor through the puncture hole. Generally, the rod (8×1 mm) was divided into three pieces and inserted in three different positions. Control mice were sham irradiated. Fractionated doses were delivered at 24-h intervals. Tumor measurements were made three times a week, and the mice were sacrificed when the end point ($4 \times$ initial tumor volume) was reached.

Excision Assays. Tumors were removed under aseptic conditions, and a single cell suspension was prepared. The tumor was minced with scalpel blades and disaggregated by incubation with stirring in HBSS with .02% collagenase, .02% DNase, and .05% Proteinase K. Cell aggregates were removed by filtration through gauze, and the number of viable cells were counted on the basis of trypan blue exclusion. For preparation of radiation survival curves from tumor cells irradiated *in vitro* cells were diluted to 5×10^4 cells/ml and irradiated in suspension on ice with a range of doses. After irradiation, the cells were diluted and appropriate numbers were plated for determination of clonogenic survival. Radiation survival curves were fitted to the Linear Quadratic model by nonlinear regression analysis.

Estimation of BrdUrd Incorporation by Flow Cytometry. Single-cell suspensions prepared as described above were processed for BrdUrd-FITC direct staining, as described in the Becton Dickinson protocol, and counterstained with propidium iodide. Analysis was done using a Becton Dickinson fluorescence-activated cell analyzer with Cellfit software. Cell suspensions were prepared from tumors at intervals after implant of polymer/BrdUrd. A control sample, which had not been exposed to BrdUrd, was processed for anti-BrdUrd-FITC staining to determine the level of nonspecific antibody binding. The channel number, above which there was only 1% of labeled cells, was taken to be the lower limit of anti-BrdUrd staining, and the proportion of

cells registering beyond this channel was taken to correspond to the BrdUrd labeling index (24).

RESULTS

In Vitro Degradation. Polymer rods of 1.0 mm diameter containing 20% BrdUrd by weight were prepared and degraded as described above. The sample aliquots were analyzed for BrdUrd, and the results expressed as a percentage of the total (Fig. 1). Approximately 50% of the BrdUrd was released after 24 h and the remainder over the next 5 days. A similar pattern of drug release was seen in earlier studies using polymer rods of the same dimensions incorporating 17% cisplatinum (w/w; 22).

In Vitro Radiosensitization of RIF-1 Cells by BrdUrd. Exponentially growing cells were cultured in control culture medium or in medium containing 10^{-5} M or 10^{-4} M BrdUrd for 4 days (equivalent to approximately four population doubling times) before irradiation. Immediately after radiation, the semiconfluent cell monolayers were suspended by trypsinization and appropriate cell numbers were plated for determination of survival by clonogenic assay. Radiation survival curves were fitted using the linear quadratic model (Fig. 2), and survival parameters are shown in Table 1. A BrdUrd concentration in the medium of 10^{-4} M produced radiosensitization characterized by an increase in the size of the α constant of the linear quadratic model.

In Vivo Radiosensitization of RIF-1 Cells by BrdUrd. Polymer containing 20% BrdUrd (w/w) was implanted in RIF-1 tumors, as described. At intervals after implant, tumor cell suspensions were prepared by the excision assay procedure and irradiated *in vitro*. This approach was used to determine the degree of intrinsic radiosensitization attributable to BrdUrd incorporated *in vivo*, while avoiding the input of tumor environmental factors such as hypoxia, which could influence *in situ* radioresponse. Tumors were excised, and cells were prepared at intervals after BrdUrd/polymer implant. At 3 days after implant, the radiation survival curve resembled that of the control. At 5, 8, and 10 days after implant, however, there was a significant radiosensitization again associated with an increase in α that remained at approximately the same level over the 5-day period. Representative survival curves for excised cells irradiated *in vitro* are shown in Fig. 3, and survival parameters are shown in Table 1.

Proportion of Cells Incorporating BrdUrd in Vivo. Tumor cell samples were prepared at intervals after polymer/BrdUrd implant, and assayed for BrdUrd incorporation by flow cytometry. The results, shown in Table 2, indicate that between 3 and 8 days after implant 40 to 50% of cells were positive for BrdUrd, whereas by 10 days the proportion had declined to 20%. Also shown in Table 2 are cell cycle

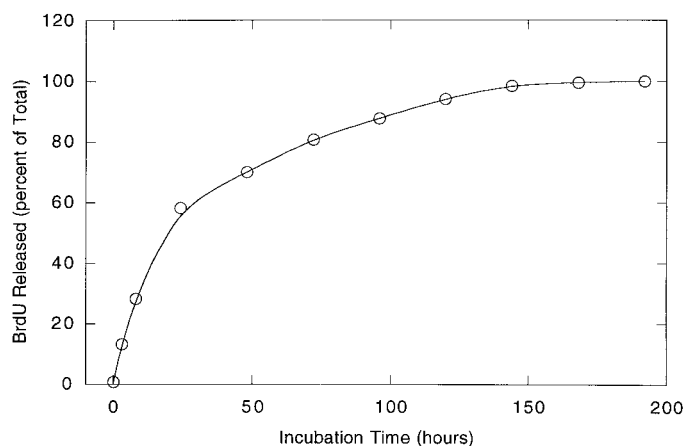


Fig. 1. Release of BrdUrd from polymer rod *in vitro*.

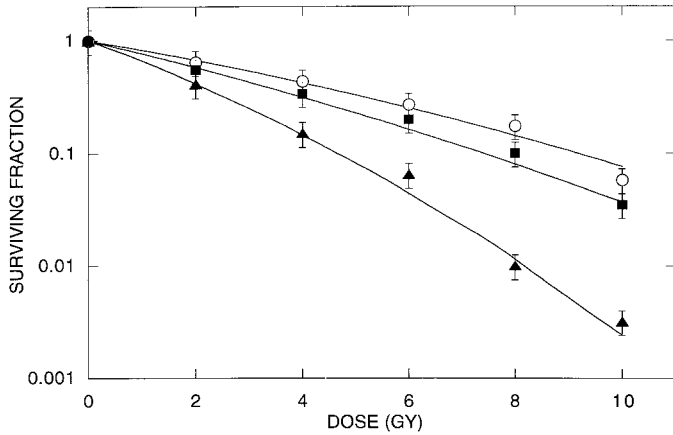


Fig. 2. Radiation survival curves for RIF-1 cells exposed for 96 h to different levels of BrdUrd in the medium. ○, control; ■, 10⁻⁵ M BrdUrd; ▲, 10⁻⁴ M BrdUrd. Bars, SD.

Table 1 Radiation survival parameters for RIF-1 cells exposed to BrdUrd in vivo and in vitro

BrdUrd concentration in the medium	α Gy ⁻¹	β Gy ⁻²	SF ₂ ^a
0	0.196 ± 0.017 ^b	0.0045 ± 0.0030	0.66
10 ⁻⁵ M	0.279 ± 0.019 ^c	0.0007 ± 0.0036	0.57
10 ⁻⁴ M	0.423 ± 0.015 ^d	0.0095 ± 0.0044	0.40
Days of BrdUrd exposure cisplatinium			
0	0.15 ± 0.0024	0.006 ± 0.0004	0.73
5	0.22 ± 0.0076 ^e	0.005 ± 0.0014	0.63

^a Surviving fraction at 2 Gy.

^b Mean of three experiments ± SD.

^c Very significant difference from α for 0M BrdUrd in medium ($P = 0.005$).

^d Extremely significant difference from α for 0M BrdUrd in medium ($P < 0.005$).

^e Extremely significant difference from α for 0 days exposure to BrdUrd ($P = 0.001$).

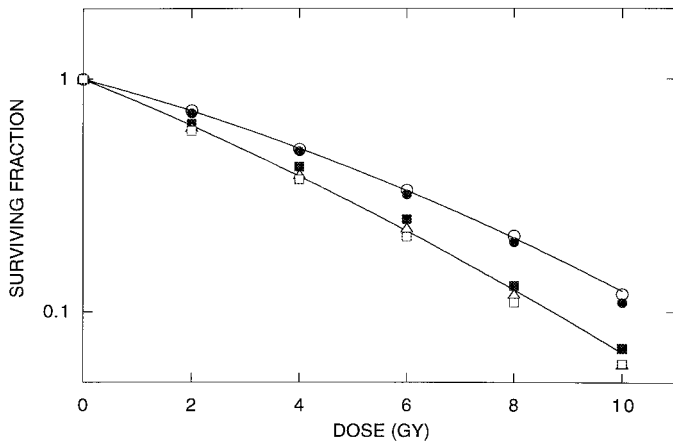


Fig. 3. Radiation survival curves for RIF-1 cells irradiated *in vitro* after exposure to BrdUrd *in vivo* for different lengths of time. ○, 0 days; ●, 3 days; ■, 5 days; △, 8 days; □, 10 days. Lines, are fitted to data points for 0 and 8 days using the linear quadratic model.

parameters for tumor cells excised and assayed at various times during tumor growth. The proportion of cells in S phase is greatest in tumors excised at 10 days of tumor growth, which would correspond to 3 days after polymer/drug implant. At this time, the proportion of cells in S phase is 12%, ~60% of that seen for exponentially growing RIF-1 cells in tissue culture. At later times, the size of the S phase compartment decreases as the tumor size increases, indicative of a decline in the size of the growth fraction. The decreasing proliferative rate may be one factor contributing to the fact that the level of BrdUrd labeling does not increase beyond 5 days of polymer/drug implant and actually falls at later times. Cell kinetic studies of the RIF-1 tumor in the early

stages of growth (100–200 mg) reported a cell loss factor of 0.54 with cells being lost from both proliferative and nonproliferative fractions (25). Cell loss from labeled and nonlabeled compartments may act to selectively reduce the number of labeled cells, and it has also been reported that the presence of BrdUrd can inhibit the incorporation of more analogue (26).

Tumor Treatment Studies. The results of experiments in which tumors were treated with radiation after implantation of polymer/BrdUrd and the time required for the tumor to regrow was measured are shown in Table 3 and Figs. 4 and 5. Implantation of polymer alone or polymer loaded with 20% or 30% BrdUrd, without irradiation, did not effect the rate of tumor growth. For treatment with a single

Table 2 Cell cycle distribution for RIF-1 cells grown in tissue culture or in vivo as s.c. tumors and the percentage of BrdUrd-positive cells at intervals after polymer implant

Tumor volume (mm ³)	Time after polymer implant (days)	% G ₀ -G ₁	% S	% G ₂ -M	BrdUrd-positive cells
RIF-1 in tissue culture		74.3	19.4	6.3	
100	3	74.5	12.0	13.5	41
200	5	76.3	8.4	15.4	53
400	8	87.1	7.6	5.3	43
600	10	86.3	4.4	9.3	28

Table 3 TGD after radiation. Effect of polymer implant, interval between polymer implant and radiation, and concentration of BrdUrd

Radiation dose (Gy)	Interval between polymer implant and radiation (days)	Concentration BrdUrd (%)	TGD (days)	SD
0			5.7	0.7
0		Polymer only	6.5	0.7
0		20	6.0	0.9
0		30	6.2	0.8
16.5			16.2	2.5
16.5	1	20	16.0	2.1
16.5	3	20	18.3	3.3
5 × 6			22.5	1.0
5 × 6	1	20	22.4	0.8
5 × 6	3	20	29.0 ^a	4.0
5 × 6	1	30	22.5	1.0
5 × 6	3	30	29.2 ^a	4.1
10 × 6			45.8	2.7
10 × 6	1	20	49.1	4.5
10 × 6	3	20	76.9 ^b	6.0
10 × 6	1	30	53.3 ^c	8.2
10 × 6	3	30	77.7 ^b	6.7

^a Very significant difference from TGD for tumor without implant ($P = 0.0078$).

^b Extremely significant difference from TGD for tumor without implant ($P < 0.0001$).

^c Difference from TGD for tumor without implant not quite significant ($P = 0.088$).

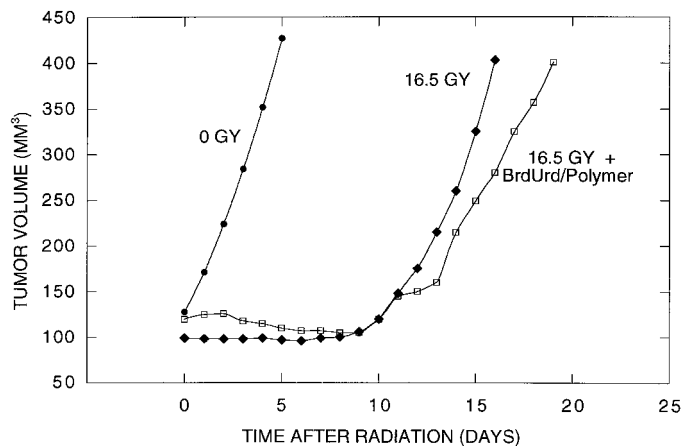


Fig. 4. Tumor volume after acute radiation exposure. ■, 0 Gy; ◆, 16.5 Gy; □, 16.5 Gy + BrdUrd/polymer implanted 3 days before irradiation.

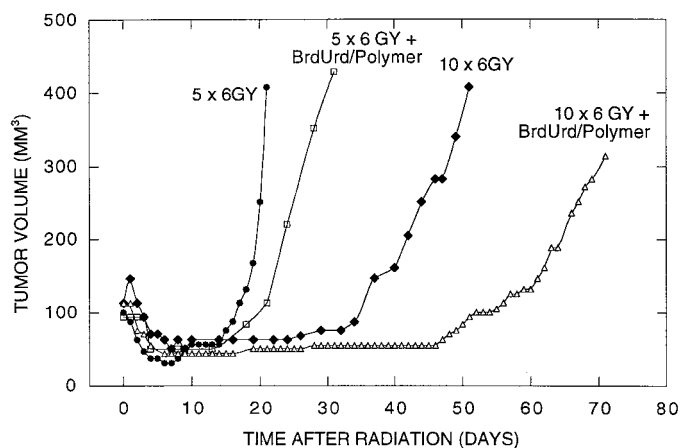


Fig. 5. Tumor volume after treatment with fractionated radiation. ●, 5 × 6 Gy; □, 5 × 6 Gy + BrdUrd/polymer; ◆, 10 × 6 Gy; ◇, 10 × 6 Gy + polymer/BrdUrd. Polymers were implanted 3 days before the first radiation fraction. Experimental groups consisted of five to eight mice. SDs of the mean volumes, which were calculated for each time interval, did not exceed 10% of the mean. Error bars are omitted for clarity.

radiation dose, no radiosensitization was seen when BrdUrd was implanted 1 day before radiation. When the interval between implant and radiation exposure was increased to 3 days there was a small increase in TGD associated with polymer implant which was not statistically significant. Tumors treated with 5 or 10 dose fractions were radiosensitized, but only if the polymer was implanted 3 days before the first radiation dose. Even for prolonged radiation treatments it seems to be necessary, if radiosensitization is to occur, to have a period of time preradiation, during which BrdUrd incorporation can take place. The most effective treatment was 10 radiation fractions combined with BrdUrd implant 3 days before the first fraction (in this case the period of growth delay) for mice in whom the tumor eventually regrew was almost 80 days compared with 45 days for radiation treatment alone. In the polymer-implanted 10-fraction group, two of eight mice were cured of the tumor (no regrowth seen for 120 days), whereas no cures were seen for the radiation only group. These results are summarized in Fig. 6. The effect of loading the polymer with different concentrations of BrdUrd was also investigated. There was no significant difference, in terms of TGD, between the response to implant of polymers loaded with 20% BrdUrd and those loaded with 30% BrdUrd for any of the treatment groups.

DISCUSSION

Results of *in vitro* measurements of the release of BrdUrd from the CCP:SA polymer followed the pattern common to a number of other polymer drug release studies (22, 27) in that there was an initial period of rapid release over the first 24 h, followed by a period of slow release lasting for ~150 h. In a study by Williams *et al.* (18), in which the rate of IdUrd release from a PCCP:SA disc (10 mg weight and 2.5 mm diameter) was measured *in vitro*, it was found that the release rate was proportional to the amount of the compound loaded into the polymer and could also be increased by co-loading with another hydrophilic component, such as D-glucose. Studies with cisplatin have shown that the rate of drug release is also proportional to the size of the polymer fragment (22) and presumably similar factors influence the release of BrdUrd.

It was shown in *in vitro* experiments that the extent of radiosensitization when BrdUrd was present in the medium for approximately three doubling times was dose-dependent and related to an increase in the size of the α component of the linear quadratic model. RIF-1 tumor cells, which were allowed to incorporate BrdUrd *in vivo* and

were irradiated *in vitro*, showed a similar pattern of response. Maximum radiosensitization was not seen until 5 days after polymer implant, indicating that an effective intratumoral BrdUrd concentration must be built up by release from the polymer and then be present for several cell doublings to effectively radiosensitize. At longer times after polymer implant, the degree of sensitization for tumor cells irradiated *in vitro* did not change further, being the same for tumors excised at 5, 8, and 10 days after implant. These results are consistent with the finding that the proportion of tumor cells that have incorporated BrdUrd reach their maximum level at 5 days after polymer implant.

There are a number of studies in the literature of labeling by thymidine analogues of tumor cells under experimental or clinical conditions. For HCT-116, a xenografted human colon tumor the fraction of labeled cells rose to 90% after perfusion with IdUrd by osmotic pump at 100 mg/kg/day (10). In a study of patients treated by i.v. infusion of IdUrd, <25% of cells of high-grade glioma cells had incorporated the analogue over 5–7 days of infusion compared with 63–85% of cells from head and neck biopsies and 57–79% of cells from high-grade sarcomas (9). The use of the PCCP:SA polymer for delivery of IdUrd to human U251 glioblastoma xenografts has been reported (28). For flank tumors, intratumoral implant with polymer containing 50% IdUrd resulted in labeling of 46% and 41% of cells at 4 and 8 days, respectively, after polymer implantation, whereas for intracranial tumors the proportion of cells labeled at 4 and 8 days after implant was 34% and 35%, respectively. The fact that for intratumoral delivery of IdUrd (28) or BrdUrd (this study) the proportion of labeled tumor cells was no >50% cannot be attributed to lack of availability of the drug. In the experiments described here, the dose of BrdUrd that could be delivered by the polymer was ~800 μ g to the tumor over 6 days. This is much more than could be delivered by, for instance, perfusion from a mini-osmotic pump, in which the 100 μ g/day that can be delivered systemically is distributed throughout the body.

It is more probable that differences in thymidine analogue incorporation result from the cell kinetic characteristics of the respective tumors. The human colon cancer xenografts were reported to be typical carcinomas with high cell loss factor and a high cell growth fraction consistent with the high cell labeling level (10). For the RIF-1 tumor, based on the proportion of cells in S phase, the growth fraction at 3 days after polymer implant has decreased by almost a factor of 2 from that seen in tissue culture, where presumably all of the cells are in cycle, and by a factor of 5 by 10 days of tumor growth. An

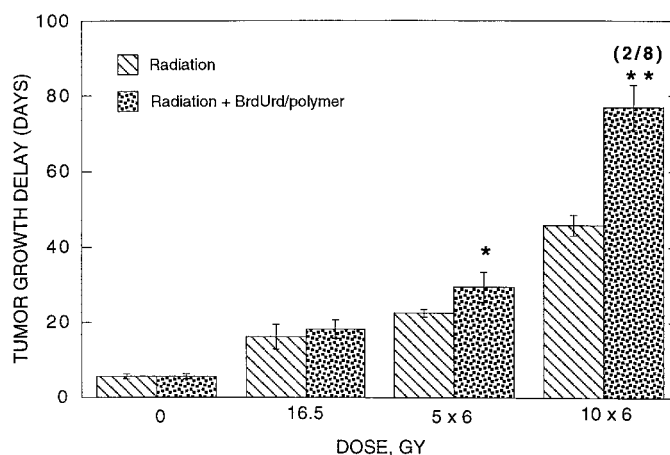


Fig. 6. Summary of results for growth delay obtained with the most effective radiation and radiation/polymer treatments. The number in parenthesis indicates the number of mice cured. *, very significant difference from TGD for tumor without implant; **, extremely significant difference from TGD for tumor without implant.

important target of radiation therapy are cells in the growth fraction, which are also those that incorporate BrdUrd, and, in terms of tumor response to treatment, the results suggest that implanted BrdUrd is reaching a high proportion of the target cell population, particularly for multifraction treatments.

The results described in this study and those reported by Williams *et al.* (18) are the only two reports in the recent literature of experimental studies of radiosensitization by halogenated pyrimidines, which have used tumor control in an animal model as an end point. In both cases, drug delivery was by an intratumoral biodegradable polymer implant and radiosensitization was observed in terms of increase in TGD and, in the present study, of tumor control. The success of the stratagem in these preclinical models suggests that this approach holds promise for transfer to the clinical setting.

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