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MAJOR COMPOUND-DEPENDENT VARIATIONS OF $^{10}B(n,\alpha)^7Li$ RBE FOR THE 9L RAT GLIOSARCOMA *IN VITRO* AND *IN VIVO*.

J.A. Coderre, M.S. Makar, P.L. Micca, M.M. Nawrocky, D.D. Joel and D.N. Slatkin

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Medical Department, Brookhaven National Laboratory, Upton, NY 11973

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ABSTRACT

Relative biological effectiveness (RBE) values for the high linear-energy-transfer (LET) radiations produced during boron neutron capture therapy (BNCT) were determined using the 9L rat gliosarcoma both *in vitro* and as an intracerebral tumor. In the absence of ^{10}B, the combined effect of the recoiling protons from the $^{14}N(n,p)^{14}C$ and the $^1H(n,n')p$ reactions, compared to an iso-effect endpoint produced by 250 kVp x-rays, yielded RBEs for these high-LET protons of 4.4 *in vitro* and 3.8 in an *in vivo/in vitro* assay.

RBEs for the $^{10}B(n,\alpha)^7Li$ reaction were calculated from cell survival data following reactor irradiation in the presence or in the absence of either the amino acid, *p*-boronophenylalanine (BPA) or the sulfhydryl dodecaborane dimer (BSSB). With BPA, RBE values ranged from 3.5 to 11.4, while under the same set of conditions with BSSB, RBE values ranged from 1.1 to 4.3. *In vitro*, higher RBEs for the $^{10}B(n,\alpha)^7Li$ reaction using BPA than with BSSB suggest a difference in distribution of ^{10}B relative to the nucleus. The calculated RBE values for BSSB-based BNCT, using an *in vivo/in vitro* assay, were physically unrealistic (1.1-1.4). The results suggest that the tumor cell survival endpoint is inappropriate for BSSB and that a different population of cells (such as vascular endothelium) may be the target during BSSB-based BNCT of intracerebral tumors.

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INTRODUCTION

Studies in rats bearing transplanted cerebral gliosarcomas have shown that the sulfhydryl borane dimer (BSSB) [1] and the amino acid *p*-boronophenylalanine (BPA) [2], both produce about the same level of ^{10}B in the tumor but produce considerably different amounts of ^{10}B in the blood and normal brain. Long-term survival of rats bearing intracerebral 9L gliosarcomas has been reported following BNCT at the Brookhaven Medical Research Reactor (BMRR) with each of these compounds [1,2]. To compare BNCT doses with conventional photon irradiation, it is useful to use multiplicative factors (RBEs) for the high-LET components of the BNCT dose and to express the total BNCT dose as the sum of RBE-corrected components with a unit of Gy-equivalent (Gy-Eq). RBE values of 2.0 for fast neutrons and 2.0 for the neutron capture reaction in nitrogen have generally been assumed based on theoretical considerations of the proton energies involved [3]. Recently, it has been proposed that a value of 1.6 be adopted for the fast neutron component of reactor derived neutron beams [4]. RBE values reported for the $^{10}B(n,\alpha)^7Li$ reaction, using a variety of *in vitro* [5-7] and *in vivo* [8-10] endpoints, have generally been in the range of 2.0 to 3.7. A value commonly assumed by us and others for the RBE of the $^{10}B(n,\alpha)^7Li$ reaction is 2.3.

In this report, tumor cell survival data obtained *in vitro* or *in vivo/in vitro* has been used to calculate unique sets of RBE values for each boron compound that are consistent with the effects produced with 250 kVp x-rays but that differ considerably from the previously assumed values. The RBE values for the $^{10}B(n,\alpha)^7Li$ reaction products were highly compound dependent. The cell survival data and the calculated RBE values suggest that the similar efficacies of these two compounds *in vivo* (long-term survival) are mediated by different mechanisms at the cellular level, most probably related to the microdistribution of ^{10}B. This report represents the first example of a predictive assay (clonogenic tumor cell survival) that is clearly unpredictive for a BNCT boron-delivery agent (BSSB).

MATERIALS AND METHODS

The GS-9L rat gliosarcoma cell line was maintained in DMEM medium supplemented with 5% fetal bovine serum (inactivated). For initiation of brain tumors, 10^4 cultured cells in 1 μl of medium were injected into the left frontal lobe of male Fisher 344 rats as previously described [1,11]. Boron analysis was performed by measuring the 478 keV prompt-gamma photons produced during $^{10}B(n,\alpha)^7Li$ reactions [12]. BPA, as the 95% ^{10}B-enriched mixture of the D and L enantiomers, was purchased from the Callery Chemical Co., Pittsburgh, PA. BPA was administered orally, in two doses three hours apart, by intubation of unanesthetized rats using 3-ml doses of an aqueous slurry of BPA at neutral pH that delivered 1500 mg of D,L-BPA/kg body weight per dose. Reactor irradiation was carried out five hours after the second dose when ^{10}B concentrations in tumor, blood and normal brain were 39, 12 and 10 μg ^{10}B/g, respectively [2]. BSSB was prepared and infused as described by Joel et al. [1]. The infusion schedule produced average ^{10}B concentrations in tumor, blood and normal brain of 37, 49 and 1 μg ^{10}B/g, respectively. Reactor irradiation was begun within 15 minutes after the termination of the infusion.

BNCT *in vitro*.

Irradiation of GS-9L cells *in vitro* was carried out as previously described [6,13]. Before irradiation, cells were preincubated for 18 hours with BPA or BSSB at a concentration of approximately 25 μg ^{10}B/ml of growth medium. The same concentration of ^{10}B was maintained in the medium during trypsinization and harvesting, as well as during the reactor irradiation. Following the irradiation, the cells were diluted with ^{10}B-free medium and plated into petri dishes for colony-forming survival assay. For each experiment, two or three different dilutions were used per tube with five replicate dishes per dilution. After two weeks, the plates were washed with HBSS, fixed with absolute ethanol and stained with 10% Giemsa. Colonies with > 50 cells each were counted. Colony-forming efficiency on control

plates was approximately 70 to 85% of the unirradiated, viable (Trypan-Blue-excluding) cells plated. Survival fraction was expressed as percentage of the colony counts on control plates.

Clonogenic cell survival following BNCT *in vivo*.

Rat brain tumor irradiations were carried out as described [1,2,14]. Within 5 minutes after the reactor irradiation the rats were euthanized and the intracerebral gliosarcomas were removed aseptically, minced and incubated with trypsin-EDTA (0.05% trypsin-0.53 mM EDTA in HBSS, without calcium or magnesium) for 30 min at 37°C. Fragments of tumor tissue were removed by centrifugation. Aliquots of the single-cell suspension obtained from the disaggregated tumor were diluted and plated for colony-forming assay. For each tumor, two or three dilutions were used with five replicate dishes per dilution. The plating efficiency of similarly-treated control tumors was approximately 50 - 60%, based on viable (Trypan Blue-excluding) cells plated. Survival fraction in the BNCT-treated tumors was normalized to the plating efficiency of the control tumors.

X-ray irradiations.

In vitro and *in vivo* irradiations with 250 kVp x-rays were carried out using a General Electric Maxitron 250 (250 kVp, 30 mA, 0.5mm Cu and 1.0mm Al filtration). The dose rate to cells *in vitro* was approximately 90 cGy/min. Irradiations of tumor-bearing rats with 250 kVp x-rays utilized the body shield and holder previously described [1]. The dose rate at the head surface was approximately 3.75 Gy/min.

RESULTS AND DISCUSSION

Dosimetry.

The dosimetric parameters of the *in vitro* irradiation geometry have been reported previously [13]. The average thermal neutron (n_{th}) fluence rate inside the irradiation tubes, determined by gold foil

activation, was 4.7 x 10^9 $n_{th}cm^{-2}s^{-1}$ at 1 MW reactor power. Tumor-bearing rats were irradiated at 1.25 MW reactor power [1,2]. The thermal neutron flux was 9.8 x 10^9 $n_{th}cm^{-2}s^{-1}$ at the head surface and 6.5 x 10^9 $n_{th}cm^{-2}s^{-1}$ at the center of the tumor 4 mm beneath the skull. Table 1 lists the physical dose rates (Gy/MW-min) of the dosimetrically significant beam components for both *in vitro* and *in vivo* irradiations.

Table 1. Dose components during BMRR thermal neutron beam irradiations. All doses are expressed as Gy/MW-min with no RBE correction.

Dose Component	*in vitro*	*in vivo*
$^{10}B(n,\alpha)^7Li$	0.024	0.034
	(per μg ^{10}B/ml)	(per μg ^{10}B/g)
fast neutrons	0.13	0.27
$^{14}N(n,p)^{14}C$	0.031	0.076
total gamma	0.06	0.11

Tumor cell survival *in vitro*.

Cell survival following *in vitro* irradiation in the thermal beam of the BMRR in the presence of BPA, BSSB or no added boron compound is shown in Figure 1 plotted *versus* physical dose (Gy). The average ^{10}B concentration in the medium during the irradiation for the BPA experiments was 26.8 ± 1.5 μg ^{10}B/ml and for the BSSB experiments was 25.8 ± 2.5 μg ^{10}B/ml. The data in Figure 1 show that, at comparable physical radiation doses, the high-LET radiations associated with BMRR irradiations (in the presence or absence of boron) are more effective than 250 kVp x-rays. Moreover, at equal physical

doses, BPA-based BNCT was significantly more effective than either BSSB-based BNCT or BMRR irradiation only. BSSB-based BNCT was not significantly different than BMRR irradiation only.

Tumor cell survival *in vivo*.

Figure 2 shows the surviving fraction of clonogenic gliosarcoma cells as a function of dose (in Gy) following irradiation *in vivo* as determined by the *in vivo/in vitro* survival assay. As was observed *in vitro* (Figure 1), the high-LET radiations associated with reactor irradiations, either in the presence or absence of added boron compound, were more effective than equal physical doses (Gy) of 250 kVp x-rays. *In vivo*, at the same physical dose to the tumor, BSSB-based BNCT was less effective than either BPA-based BNCT or BMRR irradiation only.

RBEs for fast neutrons and the $^{14}N(n,p)^{14}C$ reaction.

In the absence of ^{10}B, the fast neutrons and the products of the $^{14}N(n,p)^{14}C$ reaction comprise 73% of the total dose *in vitro* and 76% of the total dose *in vivo* (see Table 1). For the fast neutron energies of interest, the dose is almost entirely due to proton recoil from the $^{1}H(n,n')p$ reaction [3]. We have chosen to assign the same RBE value to all protons, i.e., the recoiling proton from the collision of a fast neutron with hydrogen (proton energies approximately 500 keV) and to the 620 keV proton ejected following the capture of a thermal neutron by nitrogen. The RBE of the combined recoil proton dose resulting from fast neutrons and the $^{14}N(n,p)^{14}C$ reaction was calculated by direct comparison of the dose (Gy) of BMRR irradiation (in the absence of boron) required to reduce cell survival to 10%, 1.0% or 0.1% to the dose of 250 kVp x-rays required to produce the iso-effect endpoint *in vitro* (Figure 1) and *in vivo/in vitro* (Figure 2). To isolate the effect of the high-LET beam components, an amount of dose (Gy) equivalent to the gamma component of the reactor-irradiation-only dose, was subtracted from the iso-effect x-ray dose. The ratio of the remaining x-ray dose to the combined dose from the fast neutrons and the $^{14}N(n,p)^{14}C$ reaction was taken as the RBE of these high-LET protons. For example, *in vitro*, at 10% survival (Figure 1), 2.7 Gy of BMRR radiations (no boron) produced the same effect as 9.7 Gy of x-

rays. The gamma component of the BMRR-only irradiation was 27% or 0.7 Gy. Therefore, 2.0 Gy of high-LET radiations (fast neutrons and $^{14}N(n,p)^{14}C$ reaction) are equivalent to 9.0 Gy of x-rays. This yields a value of 4.5 for the RBE of these high-LET beam components. The RBE values at 10%, 1.0%, and 0.1% cell survival *in vitro* were 4.5, 4.4 and 4.3, respectively, (mean = 4.4); the 10%, 1.0% and 0.1% cell survival data *in vivo/in vitro*, yielded RBE values of 3.5, 3.9, and 4.1, respectively (mean = 3.8). These RBE values of 4.4 *in vitro* and 3.8 *in vivo/in vitro* are considerably higher than the values generally cited in the BNCT literature, but are not inconsistent with reports of the variation of neutron RBE with energy [15], or the effect of cadmium-filtered BMRR core spectrum neutrons on pig skin [16].

RBEs for the $^{10}B(n,\alpha)^{7}Li$ Reaction.

Both the *in vitro* and the *in vivo/in vitro* cell survival data presented in Figures 1 and 2 have been replotted *versus* reactor irradiation (units of MW-min) in Figures 3 and 4, respectively, for calculation of RBEs for the $^{10}B(n,\alpha)^{7}Li$ reaction. Equation 1 describes the sum of all dose components resulting from BMRR irradiation in the presence (left side of equation) or absence (right side of equation) of added boron at an iso-effect endpoint. All physical dose rates (Gy/MW-min) were measured (see Table 1); the RBE for the fast neutrons and the $^{14}N(n,p)^{14}C$ reaction was calculated (see above) from cell survival data. Equation 1 can be solved to yield the RBE of the $^{10}B(n,\alpha)^{7}Li$ reaction.

$$t_1[R_B k_B B + R_n k_n + R_p k_p + R_g k_g] = t_2[R_n k_n + R_p k_p + R_g k_g] \qquad \text{[Eq.1]}$$

In Equation 1, B is the ^{10}B concentration present at the time of irradiation (μg ^{10}B/g); k_B is the dose rate from the $^{10}B(n,\alpha)^{7}Li$ reaction (Gy/MW-min per μg ^{10}B/g); k_n, k_p, and k_g are the dose rates (Gy/MW-min) for fast neutrons, protons from the nitrogen reaction, and gamma photons, respectively (see Table 1). R_B, R_n, R_p, and R_g are RBE values for the $^{10}B(n,\alpha)^{7}Li$ reaction, fast neutrons, the $^{14}N(n,p)^{14}C$ reaction, and gamma photons, respectively. The reactor irradiation (MW-min) required to reduce colony-forming glioma cell survival fractions equally in the presence or absence of boron compound, t_1 and t_2,

respectively, was interpolated from the appropriate pair of lines in Figure 3 or Figure 4. In solving Equation 1, the mean of the RBE values derived above for the high-LET BMRR thermal beam components was used for both R_n, fast neutron RBE, and R_p, RBE of the proton from the $^{14}N(n,p)^{14}C$ reaction, (i.e., *in vitro*, $R_n = R_p = 4.4$; *in vivo/in vitro*, $R_n = R_p = 3.8$). Table 2 lists the calculated R_B values, derived from both *in vitro* (Figure 3) and *in vivo/in vitro* (Figure 4) experiments with BPA or BSSB.

Table 2. RBE Values for the $^{10}B(n,\alpha)^7Li$ Reaction at three different cell survival endpoints during BPA-based or BSSB-based BNCT.

	Endpoint	RBE *in vitro*	RBE *in vivo*
BPA	10%	11.4	4.1
	1.0%	9.4	3.6
	0.1%	8.2	3.5
BSSB	10%	4.3	1.4
	1.0%	3.7	1.1
	0.1%	3.3	1.1

RBE values for the $^{10}B(n,\alpha)^7Li$ reaction were dependent on the boron compound, on the assay system used (*in vitro* or *in vivo/in vitro*), and (to a lesser degree) on the survival fraction endpoint chosen (variance < 20%). The RBE value for BPA was consistently higher than that determined for BSSB under the same conditions and at the same ^{10}B concentration. For both compounds, the RBE

values determined *in vitro* were consistently higher than those determined *in vivo/in vitro*. The RBE values calculated for BSSB *in vivo/in vitro* are unrealistically low for the high-LET products of the $^{10}B(n,\alpha)^{7}Li$ reaction. These data suggest that the clonogenic tumor cell survival endpoint is inappropriate for BSSB. The compound-dependent differences in the RBEs for the $^{10}B(n,\alpha)^{7}Li$ reaction also explain the significant differences in cell kill for BPA-based and BSSB-based BNCT carried out at equal ^{10}B concentrations and equal physical dose (Figures 1 and 2) or equal reactor irradiation times (Figures 3 and 4).

Cell Survival *versus* RBE-corrected dose (Gy-Eq).

The wide range of values for the RBE of the $^{10}B(n,\alpha)^{7}Li$ reaction presented in Table 2 are unprecedented at both the high and the low extremes. However, all of the 9L gliosarcoma cell survival data and the derived RBE values show remarkable internal consistency. When either the *in vitro* or the *in vivo/in vitro* cell data is replotted *versus* RBE-corrected dose (Gy-Eq), using the appropriate set of RBE values derived above, the cell survival curves for BPA-based BNCT, BSSB-based BNCT, and BMRR-irradiation-only all superimpose on the x-ray survival curve. Figure 5 shows the *in vivo/in vitro* data plotted *versus* Gy-Eq.

Conditions identical to those used for the *in vivo/in vitro* survival assay in this report have previously been shown to be therapeutically effective for both BSSB-based BNCT [1] and for BPA-based BNCT [2]. The 90% tumor control doses (Gy-Eq), estimated using the RBEs derived herein, were 48 Gy-Eq for BPA and approximately 20 Gy-Eq for BSSB. A possible explanation for the apparent paradox of equal effectiveness of BPA and BSSB *in vivo* (long-term survival endpoint) in spite of significantly different tumor doses (Gy-Eq) (due to the compound dependence of the RBE value for the $^{10}B(n,\alpha)^{7}Li$ reaction), could have to do with the compound distribution at the cellular level and the radiobiological mechanisms by which the tumor cells are killed. The disparity could be ascribed to preferential absorption of BSSB in the gliosarcoma vasculature and of BPA in the neoplastic cells of the gliosarcoma. The radiations derived from the $^{10}B(n,\alpha)^{7}Li$ reaction would then act directly on the tumor

cells in BPA-based BNCT, whereas the therapeutic effectiveness of BSSB-based BNCT would be due, at least in part, to damage to the tumor vasculature due either to the selective accumulation of BSSB in the vascular endothelial cells or to the high levels of BSSB in the blood, or both. The use of a predictive assay, using tumor cell survival as the endpoint, for a boron compound (BSSB) which may kill tumor cells by an indirect method is inappropriate and, in this case, clearly unpredictive. The unrealistically low RBE values for the $^{10}B(n,\alpha)^{7}Li$ reaction with BSSB *in vivo/in vitro* (Table 2) tend to support this view. These results show that *in vivo/in vitro* clonogenic survival assays may be predictive of *in vivo* BNCT efficacy for some, but not necessarily for all, boron-delivery agents.

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FIGURE LEGENDS

Figure 1. Clonogenic survival of 9L gliosarcoma cells as a function of dose (Gy) following irradiation *in vitro* with 250 kVp x-rays, BMRR thermal neutron beam alone, or BMRR thermal neutron beam combined with the boron compounds BPA or BSSB. Data are plotted as the geometric mean (± 1 SD) of between four and ten individual experiments with five replicate dishes plated per point per experiment. The data were fit with a linear-quadratic line that was forced to pass through the origin.

Figure 2. Clonogenic survival of 9L gliosarcoma cells as a function of dose (Gy) following irradiation of intracerebral tumors with either 250 kVp x-rays, the BMRR thermal neutron beam only, or the BMRR thermal neutron beam following administration of either BPA or BSSB. Each point represents the geometric mean (± 1 SD) of the plating efficiencies at each dilution for four to six individual tumors. For the BMRR irradiations, the number of points was insufficient for curve fitting; the data points were connected with straight lines. The x-ray data were fit with a linear-quadratic line that was forced to pass through the origin.

Figure 3. Clonogenic survival of 9L gliosarcoma cells as a function of reactor exposure *in vitro* in the absence of boron or in the presence of a constant amount of boron as either BPA or BSSB. The same *in vitro* survival data shown in Figure 1 are replotted in Figure 3 versus reactor exposure (MW-min). For the *in vitro* irradiation geometry, 1 MW-min yields 2.6 x $10^{11} n_{th} cm^{-2}$.

Figure 4. Clonogenic survival of 9L cells following irradiation of intracerebral tumors plotted versus reactor exposure (MW-min). The same *in vivo/in vitro* survival data shown in Figure 2

are replotted in Figure 4 versus reactor exposure (MW-min). One MW-min corresponds to $3.9 \times 10^{11} n_{th} cm^{-2}$ at the center of the tumor.

Figure 5. Clonogenic survival of 9L cells following irradiation of intracerebral tumors plotted versus RBE-corrected dose (Gy-Eq). The same *in vivo/in vitro* survival data shown in Figures 2 and 4 are replotted in Figure 5 using the following RBE values (calculated above): fast neutrons + nitrogen reaction = 3.8; $^{10}B(n,\alpha)^{7}Li$ with BPA = 3.7; $^{10}B(n,\alpha)^{7}Li$ reaction with BSSB = 1.2.

Figure

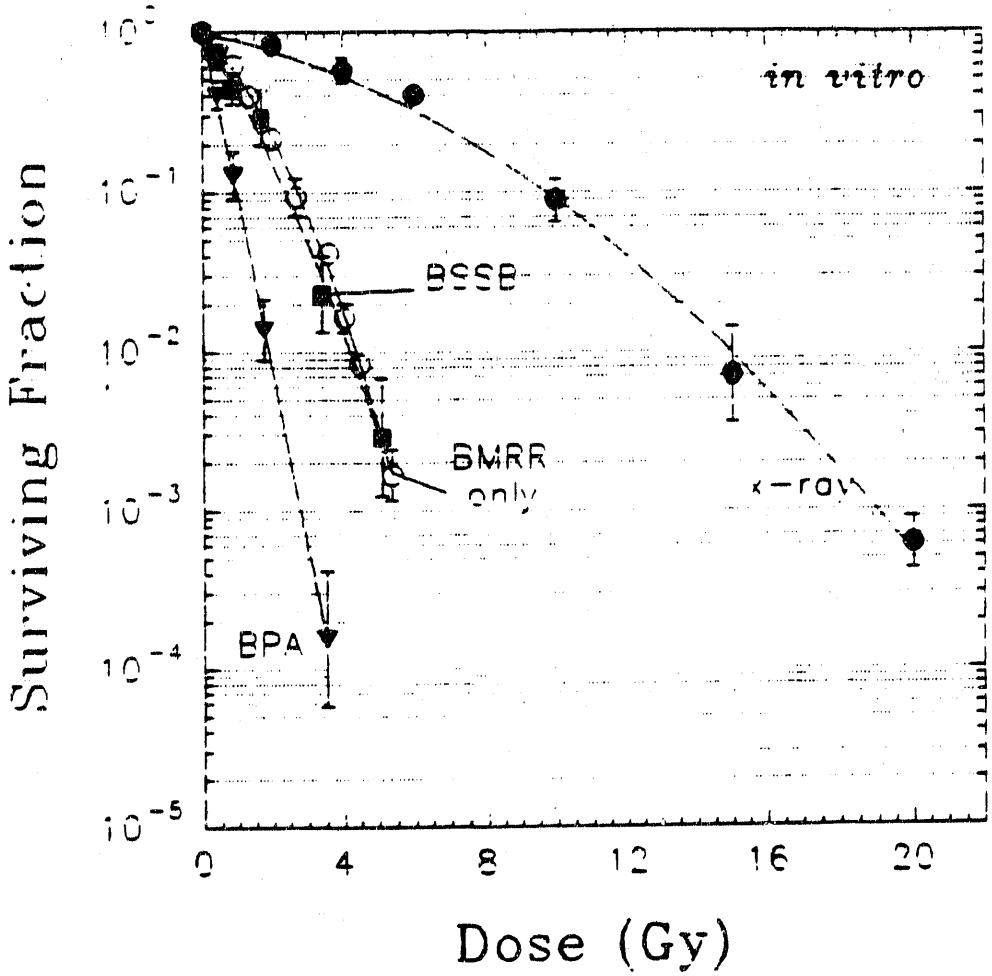
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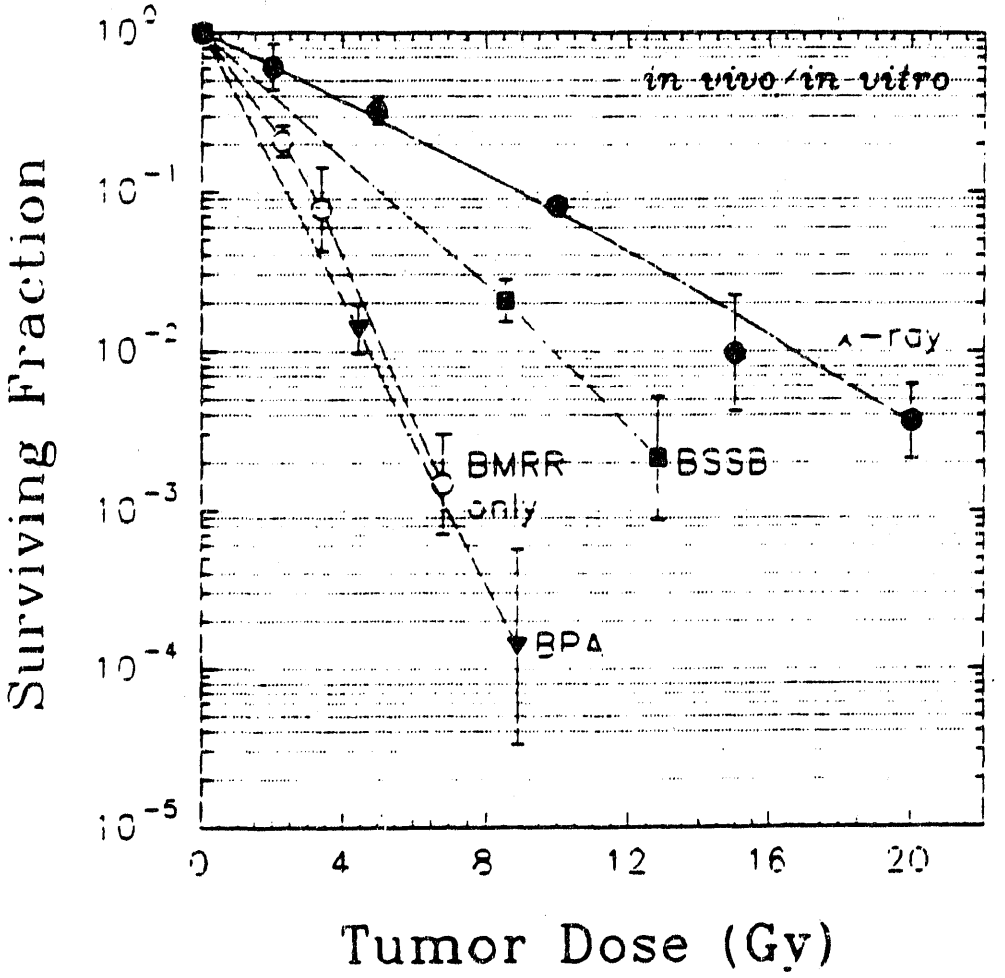
Figure 2


Surviving Fraction
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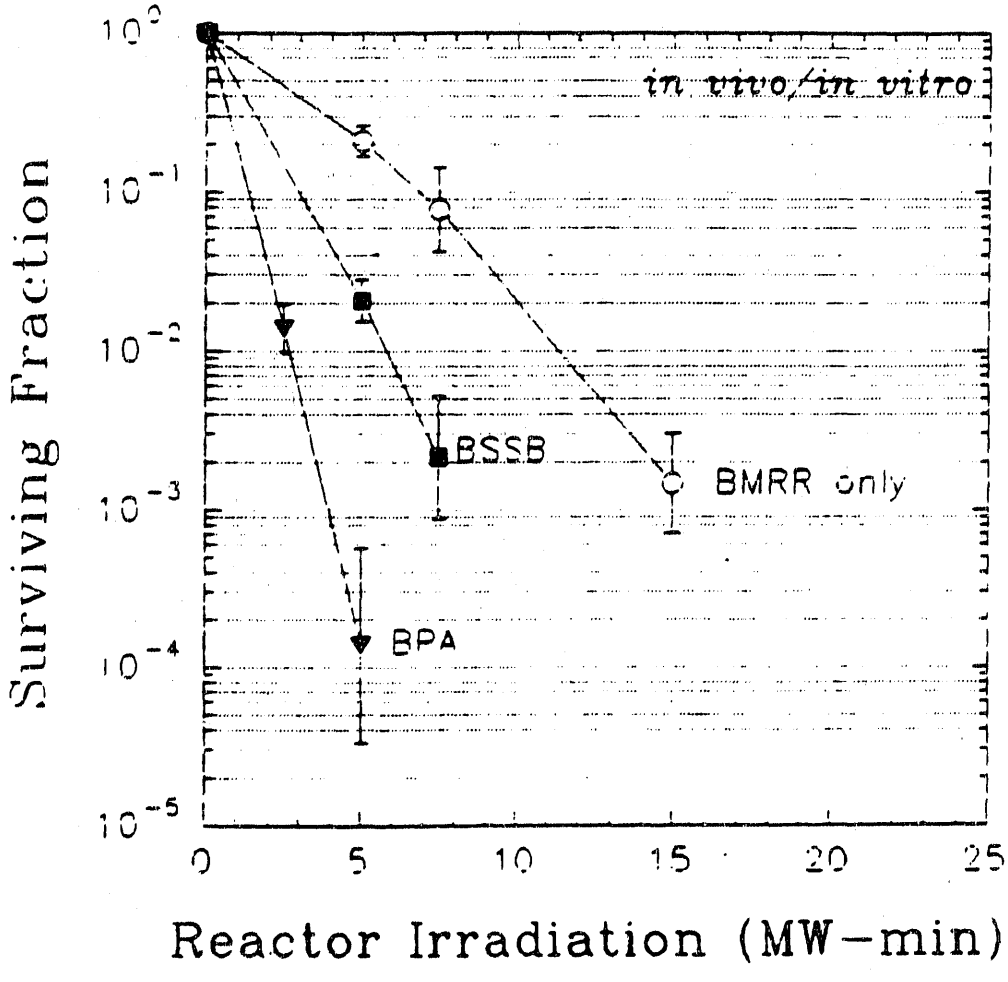
in vivo/in vitro
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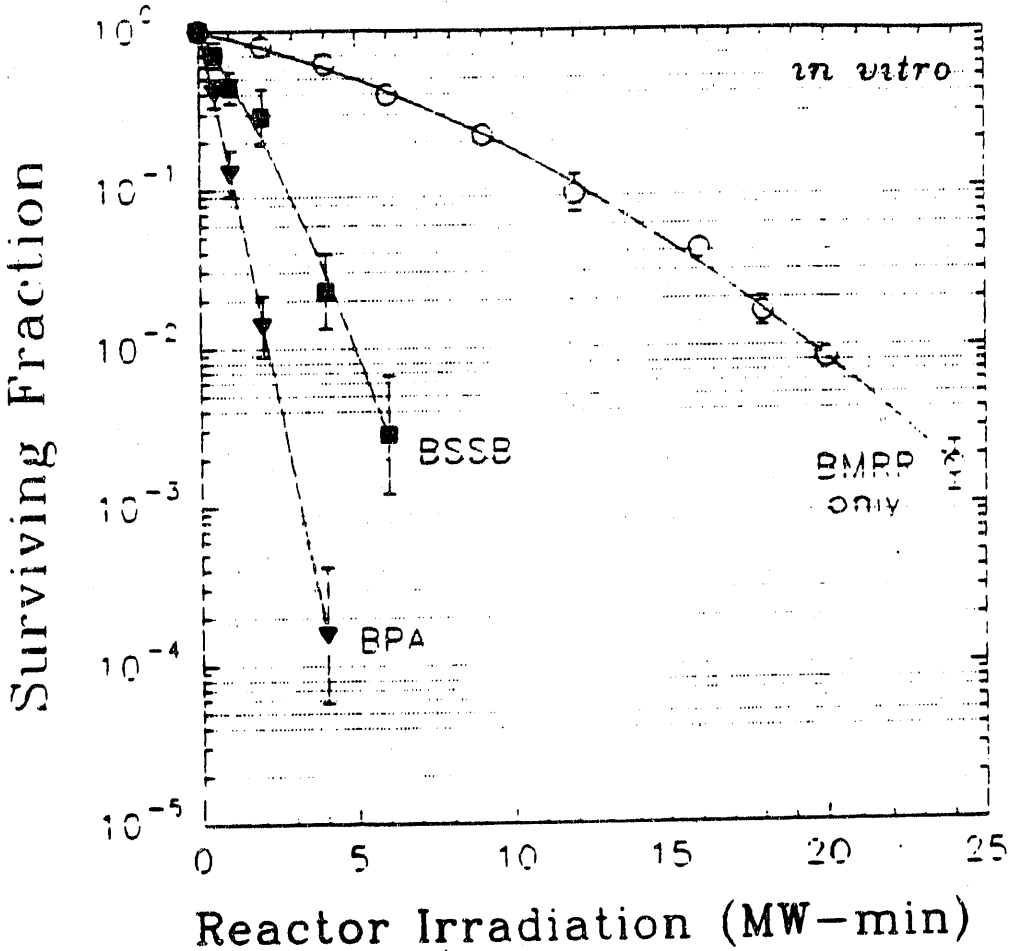
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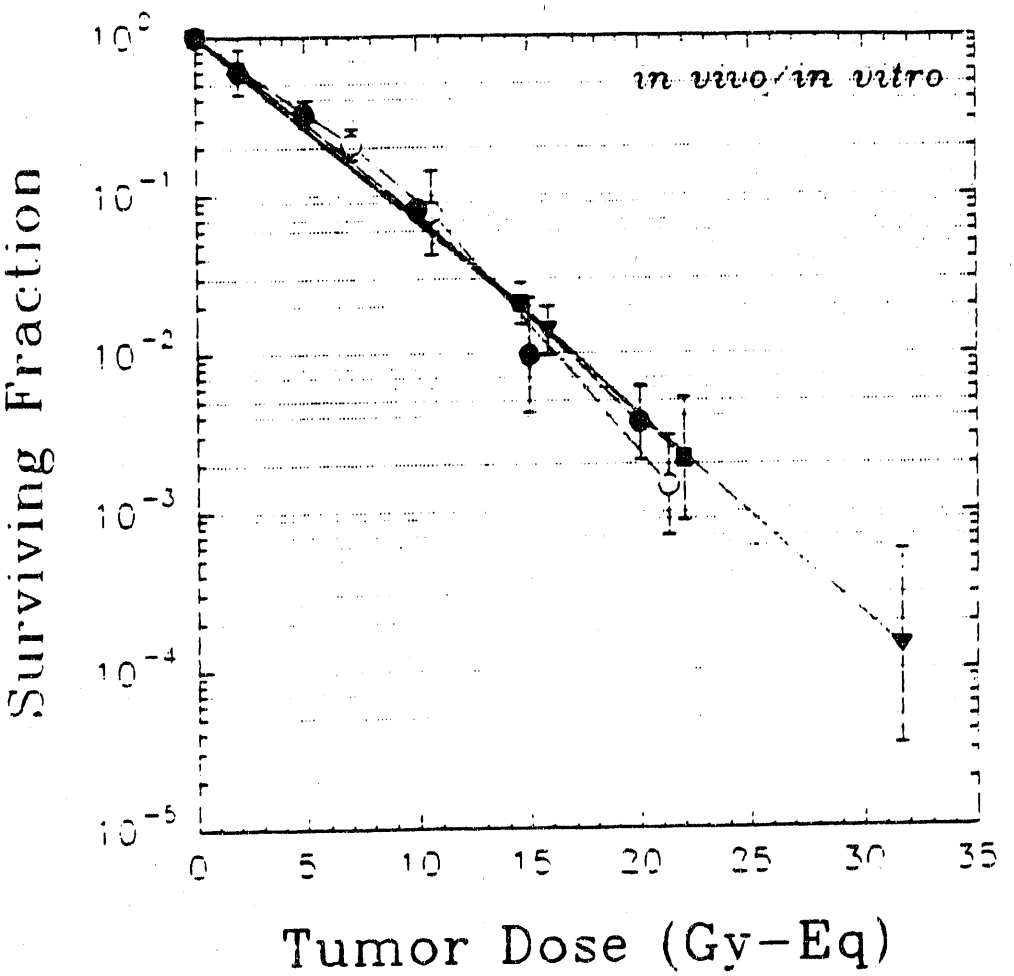
25
Reactor Irradiation (MW−min)


in vivo/in vitro
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
5
10
15
20
25
30
35
Tumor Dose (Gy-Eq)

END

DATE
FILMED
4 / 30 / 92