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Evaluation of the Potential of *p*-Boronophenylalaninol as a Boron Carrier in Boron Neutron Capture Therapy, Referring to the Effect on Intratumor Quiescent Cells

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C57BL mice bearing EL4 tumors and C3H/He mice bearing SCC VII tumors received 5-bromo-2′-deoxyuridine (BrdU) continuously for 5 days via implanted mini-osmotic pumps to label all proliferating (P) cells. Three hours after oral administration of *l-p*-boronophenylalanine-^{10}B (BPA), or 30 min after intraperitoneal injection of sodium borocaptate-^{10}B (BSH) or *l-p*-boronophenylalaninol (BPA-ol), a newly developed ^{10}B-containing α-amino alcohol, the tumors were irradiated with thermal neutron beams. For the combination with mild temperature hyperthermia (MTH) and/or tirapazamine (TPZ), the tumors were heated at 40°C for 30 min immediately before neutron exposure, and TPZ was intraperitoneally injected 30 min before irradiation. The tumors were then excised, minced and trypsinized. The tumor cell suspensions thus obtained were incubated with cytochalasin-B (a cytokinesis blocker), and the micronucleus (MN) frequency in cells without BrdU labeling (=quiescent (Q) cells) was determined using immunofluorescence staining for BrdU. Meanwhile, 6 h after irradiation, tumor cell suspensions obtained in the same manner were used for determining the apoptosis frequency in Q cells. The MN and apoptosis frequency in total (P+Q) tumor cells were determined from tumors that were not pretreated with BrdU. Without TPZ or MTH, BPA-ol increased both frequencies most markedly, especially for total cells. However, as with BPA, the sensitivity difference between total and Q cells was much larger than with BSH. On combined treatment with both MTH and TPZ, this sensitivity difference was markedly reduced, similarly to when BPA was used. MTH increased the ^{10}B uptake of all ^{10}B-compounds into both tumor cells. BPA-ol has good potential as a ^{10}B-carrier in neutron capture therapy, especially when combined with both MTH and TPZ.

Key words: Neutron capture therapy — *l-p*-Boronophenylalaninol — Quiescent cell — Tirapazamine — Mild temperature hyperthermia

A neutron capture reaction in boron (^{10}B(n,α)^{7}Li) is, in principle, very effective in destroying tumors, providing that a sufficient amount of ^{10}B can be accumulated in the target tissue and a sufficient number of very low energy thermal neutrons can be delivered there. The two particles generated in this event carry a high linear energy transfer (LET) and an average total kinetic energy of 2.34 MeV, and have a short range of approximately one cell diameter, resulting in induction of a high relative biological effectiveness (RBE).[1)]

In clinical trials of boron neutron capture therapy (BNCT) for human malignant tumors, *l-p*-boronophenylalanine-^{10}B (BPA) and sodium borocaptate-^{10}B (BSH) have been employed as ^{10}B-carriers.[1)] These compounds, however, are not ideal because of their poor tumor specificity, short retention in tumors and incomplete clearance from blood and normal tissues. Thus, new boron compounds have been investigated in an effort to improve the clinical outcome of BNCT. To develop biologically more selective BPA congeners for BNCT, *l-p*-boronophenylalaninol-^{10}B (BPA-ol) has been designed and synthesized by Kirihata *et at*.[2, 3)] BPA-ol is a ^{10}B-containing α-amino alcohol of BPA (Fig. 1).

Many tumor cells in solid tumors are non-proliferating (quiescent). Over the last 25 years, the characteristics of quiescent (Q) cells have been examined extensively, but many aspects of these cells are still uncharacterized.[4, 5)] Thus, to improve cancer treatment, the response of Q cells

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in solid tumors to various anticancer treatments should be determined, since many tumor cells are quiescent *in situ* but are still clonogenic.[4, 5] Until recently, a simple assay for assessing the response of intratumor Q cells was not available. In analyzing the response of Q cells in solid tumors, we have developed a combined method utilizing micronucleus (MN) assay and identification of proliferating (P) cells by using 5-bromo-2′-deoxyuridine (BrdU) and anti-BrdU monoclonal antibody.[6] Micronuclei have been reported to appear in dividing cells with chromosomal aberrations after irradiation. The frequency of their appearance was reported to correlate with the radiation dose and with cell killing.[7, 8] However, recent research has shown that some cells die through apoptosis after irradiation and that apoptosis after irradiation may be related to tumor sensitivity to irradiation.[9] Very recently, our method for detecting the Q cell response to irradiation using P cell labeling with BrdU and the MN frequency assay was also shown to be applicable to an apoptosis detection assay.[10]

Therefore, we evaluated the usefulness of the newly synthesized BPA-ol in BNCT from the viewpoint of the killing effect on Q tumor cells within solid tumors in comparison with the total (P+Q) tumor cell population, using our method for detecting the intratumor Q cell response in terms of the MN frequency and apoptosis frequency.[6, 10] Heat treatment at mild temperatures was reported to increase the tumor response to radiation by improving tumor oxygenation through an increase in tumor blood flow,[11] and tirapazamine (TPZ, SR-4233, WIN 59075, 3-amino-1,2,4-benzotriazine-1,4-dioxide), a well-known bioreductive agent, was reported to have highly selective cytotoxicity toward hypoxic cells in *in vivo* tumor systems.[4] Accordingly, here we also evaluated the applicability of TPZ and/or mild temperature hyperthermia (MTH) in BNCT.

MATERIALS AND METHODS

Mice and tumors SCC VII squamous cell carcinoma (Department of Radiology, Kyoto University) derived from C3H/He mice was maintained *in vitro* in Eagle's minimum essential medium. EL4 lymphoma (Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University) derived from C57BL mice was maintained *in vitro* in RPMI 1640 medium. Both media were supplemented with 12.5% fetal bovine serum. Cells were collected from exponentially growing cultures, and approximately 1.0×10^5 cells of each tumor were inoculated subcutaneously into the left hind legs of 8- to 11-week-old syngeneic female mice. Fourteen days after inoculation, each tumor had reached approximately 1 cm in diameter.

Labeling with BrdU Nine days after tumor cell inoculation, mini-osmotic pumps (Alzet model 2001, Palo Alto, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously to label all P cells for 5 days. Administration of BrdU did not change the tumor growth rate. The tumors were 1 cm in diameter on treatment. The labeling index (LI) after continuous labeling with BrdU was 55.3 (50.8–59.8) % (mean (95% confidence limit)) and 66.1 (62.3–69.9) % for SCC VII and EL4 tumor cells, respectively, and reached a plateau level at these stages. Therefore, in this study, we regarded tumor cells not incorporating BrdU after continuous labeling as Q cells.

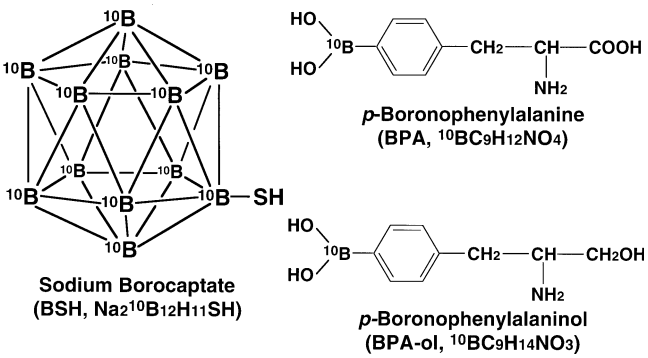

Fig. 1. Chemical structures of sodium borocaptate-^{10}B, *p*-boronophenylalanine-^{10}B and *p*-boronophenylalaninol-^{10}B.

Drug Kirihata and his colleagues synthesized BPA-ol from N-formyl-BPA ethyl ester or BPA as a starting compound through alcohol or BPA ester formation, respectively, to develop a new BPA analogue with high water solubility.[2, 3]

Hori, Nagasawa and Uto synthesized TPZ according to the established method,[12] which Brown and colleagues also employed,[13] and analyzed it by chemical ionization mass spectrometry [*m*/*z*, 179(MH+)], electron ionization mass spectrometry [*m*/*z*, 178(M+)] and infrared spectrometry [KBr]. This synthesized TPZ was regarded as identical to commonly used TPZ.

Treatment Based on the data of preliminary pharmacokinetic studies, in order to obtain similar ^{10}B concentrations at the time of neutron irradiation for each condition in both tumors, each ^{10}B-compound was administered in the following manner. The EL4 and SCC VII tumors were irradiated with a thermal neutron beam generated at the Kyoto University Reactor (KUR) 30 min after the intraperitoneal injection of BSH dissolved in physiological saline at a dose of 100 or 125 mg/kg or 3 h after oral administration of BPA dissolved in physiological saline at a dose of 750 or 1500 mg/kg, respectively. Other EL4 and SCC VII tumors were irradiated with the reactor thermal neutron beam 30 min after the intraperitoneal administration of BPA-ol dissolved in physiological saline at a dose of 300 mg/kg.

In addition, for the combination with MTH and/or TPZ, TPZ (40 mg/kg) dissolved in physiological saline was intraperitoneally injected 30 min before neutron irradiation and the tumors grown in the left hind legs of mice were heated at 40°C for 30 min by immersing the animal's foot in a water bath immediately before neutron beam exposure. We employed the same heating method as reported previously.[14] Temperatures at the tumor center equilibrated within 3 to 4 min after immersion in the water bath and remained 0.2–0.3°C below the water bath temperature. The temperature difference between the tumor center and the periphery was within 0.1°C. The water bath temperature was maintained at 0.3°C above the desired tumor temperature.

An acrylic resin instrument was made for holding 16 mice, and the tumor-bearing mice were irradiated with the neutron beam after being fixed in position with adhesive tape. A neutron beam with a cadmium (Cd) ratio of 160 was used. The Cd ratio represents the degree to which the thermal neutron beam is contaminated with fast neutrons, and refers to gold foil activation without and with Cd covers on the gold foil (Cd stops all thermal neutrons). The neutron fluence was measured from the radioactivation of the gold foil (3 mm diameter; 0.05 mm thickness) both at the front and back of the tumors. Since the tumors were small and located just beneath the surface, intratumor neutron fluence was assumed to decrease linearly from the front to back of the tumors. Thus, we used the averaged neutron fluence from the values measured at the front and back. Contaminating γ-ray doses, including secondary γ-rays, were measured with thermoluminescence dosimeter powder at the back of the tumors. For the estimation of neutron energy spectra, eight kinds of activation foil and fourteen kinds of nuclear reaction were used. Neutron absorbed dose was calculated using the flux-to-dose conversion factor.[15] Weight percentage of the tumors was assumed to be H (10.7%), C (12.1%), N (2%), O (71.4%) and others (3.8%).[16] The averaged neutron flux (n/cm^2s) and Kerma rate (cGy/h) of the employed thermal neutron beam were 2.0×10^9 and 96 for the thermal neutron range (−0.6 eV), 2.8×10^7 and 1.03 for the epithermal neutron range (0.6 eV−10 keV), and 6.6×10^6 and 28.4 for the fast neutron range (10 keV−), respectively. The contaminating γ-ray dose rate was 120 cGy/h. Each treatment group also included mice that were not pretreated with BrdU.

Some of the tumors that were not exposed to neutron beams were used to determine the ^{10}B concentration in the tumors. The ^{10}B concentration in the tumors was measured by prompt γ-ray spectrometry using a thermal neutron guide tube at KUR.[17]

Immunofluorescence staining of BrdU-labeled cells and observation of apoptosis and micronucleus formation

Based on our previous report concerning the timing of apoptosis detection,[18] a maximum value of the apoptosis frequency was observed 6 h after neutron irradiation with or without ^{10}B-compound in both EL4 and SCC VII tumor cells. Thus, 6 h after neutron exposure for the apoptosis assay, and immediately after neutron irradiation for the MN assay, tumors were excised from mice given BrdU, minced, and trypsinized. For the apoptosis assay, single cell suspensions were fixed with 70% ethanol overnight at 4°C. For the MN assay, tumor cell suspensions were incubated for 48 h in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division, and the cultures were then trypsinized and cell suspensions were fixed. For both assays, after centrifugation of fixed cell suspensions, the cell pellet was resuspended with cold Carnoy's fixative. The suspension was then placed on a glass microscope slide and the sample was dried at room temperature. The slides were treated with 2 *N* hydrochloric acid for 45 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody (Becton Dickinson, San Jose, CA) and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Sigma, St. Louis, MO). To observe double staining of tumor cells with green color-emitting FITC and red color-emitting PI (propidium iodide), cells on the slides were treated with PI and monitored under a fluorescence microscope.

The frequency of apoptosis in BrdU-unlabeled cells (=Q cells at irradiation) could be determined by counting apoptotic cells among tumor cells that showed only red fluorescence. Standard criteria for the morphological characteristics of apoptosis were chromatin condensation, nucleolar disintegration and formation of crescent caps of condensed chromatin at the nuclear periphery. Single, relatively large (≥4 μm in diameter) and roundish nuclear residues existing in extra- or intratumoral cells with intensive staining were identified as apoptotic bodies.[19, 20] The frequency was defined as the ratio of the number of apoptotic cells to the total number of observed tumor cells. The MN frequency in BrdU-unlabeled cells could be examined by counting the micronuclei in the binuclear cells that showed only red fluorescence. The MN frequency was defined as the ratio of the number of micronuclei in the binuclear cells to the total number of binuclear cells observed.[10, 20]

The ratios obtained in tumors not pretreated with BrdU indicated the apoptosis frequency and the MN frequency at all phases in the total (P+Q) tumor cell populations. More than 400 tumor cells and binuclear cells were counted to determine the apoptosis frequency and the MN frequency, respectively.

Three mice were used to assess each set of conditions and each experiment was repeated 3 times. To examine the

Table I. Micronucleus Frequency and Apoptosis Frequency at 0 Gy

Treatment	Total tumor cells	Quiescent cells
<Micronucleus frequency>		
EL4		
No treatment	0.021 (0.010–0.032)[a)]	0.025 (0.013–0.037)
BSH	0.063 (0.057–0.069)	0.079 (0.071–0.087)
BSH+heating	0.071 (0.064–0.078)	0.143 (0.133–0.153)
BSH+TPZ[b)]	0.091 (0.082–0.100)	0.188 (0.168–0.208)
BSH+heating +TPZ	0.118 (0.107–0.129)	0.333 (0.303–0.363)
BPA	0.071 (0.064–0.078)	0.075 (0.067–0.083)
BPA+heating	0.080 (0.071–0.089)	0.100 (0.090–0.110)
BPA+TPZ	0.111 (0.100–0.122)	0.153 (0.139–0.167)
BPA+heating +TPZ	0.154 (0.134–0.174)	0.256 (0.236–0.276)
BPA-ol	0.076 (0.069–0.083)	0.118 (0.108–0.128)
BPA-ol+heating	0.111 (0.100–0.122)	0.167 (0.149–0.185)
BPA-ol+TPZ	0.188 (0.168–0.208)	0.316 (0.286–0.346)
BPA-ol+heating +TPZ	0.235 (0.205–0.265)	0.333 (0.302–0.364)
SCC VII		
No treatment	0.025 (0.022–0.028)	0.053 (0.049–0.057)
BSH	0.034 (0.030–0.038)	0.067 (0.057–0.077)
BSH+heating	0.050 (0.045–0.055)	0.091 (0.081–0.101)
BSH+TPZ	0.110 (0.088–0.132)	0.161 (0.136–0.186)
BSH+heating +TPZ	0.150 (0.135–0.165)	0.206 (0.186–0.226)
BPA	0.048 (0.039–0.057)	0.068 (0.058–0.078)
BPA+heating	0.080 (0.071–0.089)	0.103 (0.088–0.118)
BPA+TPZ	0.098 (0.083–0.113)	0.131 (0.116–0.146)
BPA+heating +TPZ	0.105 (0.095–0.115)	0.190 (0.170–0.210)
BPA-ol	0.063 (0.055–0.071)	0.071 (0.061–0.081)
BPA-ol+heating	0.077 (0.070–0.084)	0.083 (0.073–0.093)
BPA-ol+TPZ	0.131 (0.117–0.145)	0.238 (0.213–0.263)
BPA-ol+heating +TPZ	0.161 (0.143–0.179)	0.286 (0.251–0.321)
<Apoptosis frequency>		
EL4		
No treatment	0.021 (0.017–0.025)	0.022 (0.020–0.024)
BSH	0.022 (0.015–0.029)	0.024 (0.015–0.033)
BSH+heating	0.023 (0.014–0.032)	0.029 (0.026–0.032)
BSH+TPZ	0.028 (0.025–0.031)	0.046 (0.041–0.051)
BSH+heating +TPZ	0.031 (0.027–0.035)	0.063 (0.055–0.071)
BPA	0.026 (0.023–0.029)	0.026 (0.014–0.035)
BPA+heating	0.026 (0.021–0.031)	0.027 (0.025–0.029)
BPA+TPZ	0.031 (0.020–0.042)	0.060 (0.059–0.061)
BPA+heating +TPZ	0.035 (0.020–0.050)	0.066 (0.059–0.073)
BPA-ol	0.028 (0.027–0.029)	0.034 (0.033–0.035)
BPA-ol+heating	0.026 (0.015–0.037)	0.035 (0.020–0.050)
BPA-ol+TPZ	0.037 (0.036–0.038)	0.042 (0.041–0.043)
BPA-ol+heating +TPZ	0.041 (0.026–0.056)	0.066 (0.059–0.073)

a) Numbers in parentheses are 95% confidence limits, determined using mean values, standard deviations and the number of observations.
b) Tirapazamine.

differences between pairs of values, Student's *t* test was used when the variances of the two groups could be assumed to be equal; otherwise the Welch *t* test was used. *P* values were from two-sided tests.

RESULTS

The ^{10}B concentrations for the BSH, BPA, and BPA-ol alone administration groups were 11.2±1.9, 11.6±2.2, and 9.2±1.7 μg/g in EL4 tumors, and 11.3±1.3, 10.2±2.1, and 8.9±1.2 μg/g in SCC VII tumors, respectively. There were no significant differences among them.

Table I shows the MN and apoptosis frequencies without radiation for total and Q tumor cells in each tumor. Without radiation, Q tumor cells showed comparatively higher MN and apoptosis frequencies than total tumor cells under all conditions in each tumor. When combined with TPZ, the frequencies for Q cells were significantly larger than those for total cells ($P<0.05$).

Fig. 2 shows the normalized MN (a) and apoptosis (b) frequencies for irradiation without MTH or TPZ as a function of the physically absorbed radiation dose in total and Q tumor cells within EL4 tumors. According to our previous reports,[10, 18)] the induced apoptosis frequencies for SCC VII tumor cells were much lower than those for EL4 tumor cells. Thus, just the normalized MN frequencies as

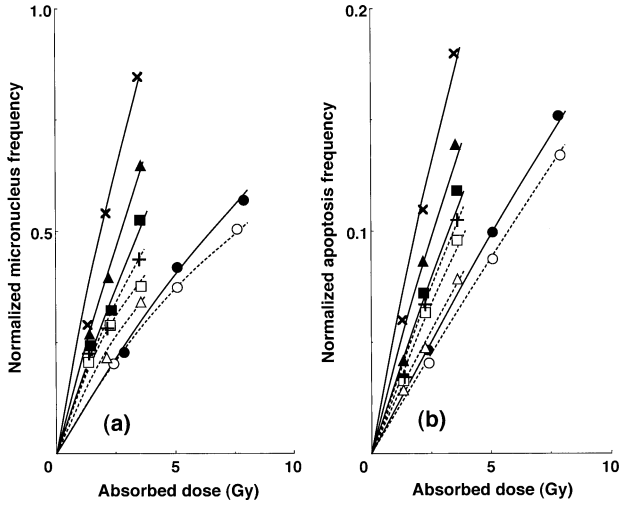

Fig. 2. Dose-response curves of normalized micronucleus frequency (a) and normalized apoptosis frequency (b) as a function of radiation dose for total (solid lines) and quiescent (dotted lines) tumor cell populations in EL4 tumors irradiated using reactor neutrons with or without a ^{10}B-compound. ●, ■, ▲, × total cells; ○, □, △, + quiescent cells. ●, ○ without ^{10}B-compound; ■, □ with BSH administration; ▲, △ with BPA administration; ×, + with BPA-ol administration. Only mean values are shown, to avoid confusion.

a function of the physically absorbed dose were examined in SCC VII tumor cells (Fig. 3). When a ^{10}B-compound was administered before neutron beam irradiation, even if no radiation was given, these frequencies were higher than when no ^{10}B-compound was administered, because of the genotoxicity of the drug (Table I). Therefore, for background correction, we used the normalized frequency to exclude the effects of the genotoxicity of the ^{10}B-compound. The normalized frequency is the frequency in the irradiated tumors minus that in the nonirradiated tumors.

With the use of ^{10}B-compounds, the normalized MN and apoptosis frequencies for each cell population in EL4 and SCC VII tumors were increased. To assess the effect of a ^{10}B-compound on the frequencies in total and Q cell populations, the enhancement ratio (ER) was calculated at various normalized frequencies using the data given in Figs. 2 and 3 (Table II). The ERs for total cells were larger than those for Q cells. BPA and BPA-ol induced significantly larger ERs for total cells than for Q cells ($P<0.05$). In total tumor cells, ER values were significantly larger for BPA and BPA-ol than for BSH ($P<0.05$), and those for BPA-ol were larger than those for BPA with a significant difference ($P<0.05$). The values were significantly larger for BSH and BPA-ol than for BPA in Q cells ($P<0.05$).

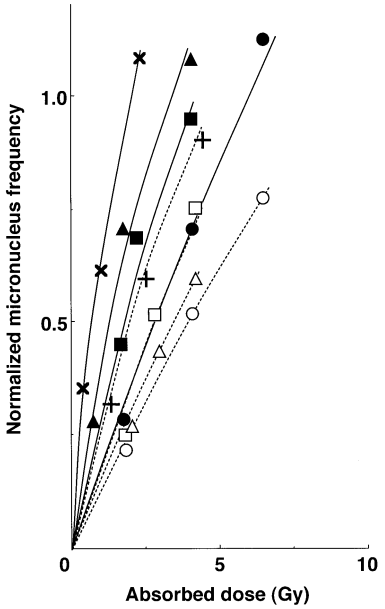

Fig. 3. Dose-response curves of normalized micronucleus frequency as a function of radiation dose for total (solid lines) and quiescent (dotted lines) tumor cell populations in SCC VII tumors irradiated using reactor neutrons with or without a ^{10}B-compound. ●, ■, ▲, × total cells; ○, □, △, + quiescent cells. ●, ○ without ^{10}B-compound; ■, □ with BSH administration; ▲, △ with BPA administration; ×, + with BPA-ol administration. Only mean values are shown, to avoid confusion.

With or without a ^{10}B-compound, the normalized MN and apoptosis frequencies were lower in Q cells than in total tumor cells. We calculated the dose-modifying factors (DMFs) of Q cells and used them to compare the radiation doses necessary to obtain various normalized frequencies in Q cells with the doses required in total tumor cells (Table III). The use of ^{10}B-compound widened the difference in sensitivity between total and Q tumor cells. Notably, BPA and BPA-ol significantly widened the sensitivity difference ($P<0.05$), compared with no ^{10}B-compound.

Figs. 4, 5, and 6 show the normalized MN frequencies in EL4 tumors, the normalized apoptosis frequencies in EL4 tumors, and the normalized MN frequencies in SCC VII tumors, for irradiation in combination with MTH and/or TPZ as a function of the physically absorbed radiation dose, respectively. In Figs. 4 through 6, the frequencies of total and Q tumor cells following neutron exposure with BSH (a), BPA (b), or BPA-ol (c) are exhibited. In both total and Q cell populations within the two tumors, after neutron irradiation with any ^{10}B-compound, the frequen-

Table II. The Effects of ^{10}B-Compounds on Each End Point[a)]

Tumor	^{10}B-Compound	End point	Total tumor cells		Quiescent cells	
<Normalized micronucleus frequency>						
EL4						
	BSH	0.3	1.85	(1.75–1.95)[b)]	1.6	(1.5–1.7)
		0.2	1.85	(1.75–1.95)	1.7	(1.65–1.75)
	BPA	0.3	2.3	(2.2–2.4)	1.25	(1.15–1.35)
		0.2	2.25	(2.15–2.35)	1.25	(1.15–1.35)
	BPA-ol	0.3	3.35	(3.2–3.5)	1.85	(1.7–2.0)
		0.2	3.25	(3.15–3.35)	2.1	(2.0–2.2)
SCC VII						
	BSH	0.75	1.45	(1.35–1.55)	1.4	(1.3–1.5)
		0.3	1.55	(1.45–1.65)	1.4	(1.3–1.5)
	BPA	0.5	1.9	(1.8–2.0)	1.15	(1.1–1.2)
		0.3	2.1	(2.0–2.2)	1.15	(1.1–1.2)
	BPA-ol	0.75	3.2	(3.0–3.4)	1.8	(1.65–1.95)
		0.3	4.0	(3.8–4.2)	2.1	(2.0–2.2)
<Normalized apoptosis frequency>						
EL4						
	BSH	0.1	1.55	(1.45–1.65)	1.45	(1.35–1.55)
		0.04	1.55	(1.45–1.65)	1.45	(1.35–1.55)
	BPA	0.06	1.9	(1.8–2.0)	1.2	(1.1–1.3)
		0.04	1.95	(1.85–2.05)	1.2	(1.1–1.3)
	BPA-ol	0.1	2.75	(2.5–3.0)	1.7	(1.6–1.8)
		0.04	2.85	(2.5–3.2)	1.75	(1.6–1.9)

a) The ratio of radiation dose required to obtain each end point without ^{10}B-compound in relation to the radiation dose required to obtain each end point with ^{10}B-compound.
b) Numbers in parentheses are 95% confidence limits, determined using mean values, standard deviations and the number of observations.

Table III. Dose Modifying Factors[a)] for Quiescent Tumor Cells Relative to Total Tumor Cell Populations

Tumor	End point	No ^{10}B-compound	BSH	BPA	BPA-ol
<Normalized micronucleus frequency>					
EL4					
	0.3	1.0 (1.0–1.05)[b)]	1.15 (1.1–1.2)	1.9 (1.85–1.95)	1.85 (1.8–1.9)
	0.2	1.0 (1.0–1.05)	1.1 (1.05–1.15)	1.85 (1.8–1.9)	1.6 (1.55–1.65)
SCC VII					
	0.5	1.4 (1.3–1.5)	1.5 (1.4–1.6)	2.35 (2.3–2.4)	2.55 (2.4–2.7)
	0.2	1.4 (1.3–1.5)	1.6 (1.5–1.7)	2.65 (2.55–2.75)	2.8 (2.7–2.9)
<Normalized apoptosis frequency>					
EL4					
	0.06	1.15 (1.1–1.2)	1.2 (1.1–1.3)	1.8 (1.7–1.9)	1.8 (1.7–1.9)
	0.04	1.1 (1.05–1.15)	1.2 (1.1–1.3)	1.8 (1.7–1.9)	1.85 (1.7–1.9)

a) The ratio of radiation dose required to obtain each end point in quiescent tumor cells in relation to the radiation dose required to obtain each end point in total tumor cells.
b) Numbers in parentheses are 95% confidence limits, determined using mean values, standard deviations and the number of observations.

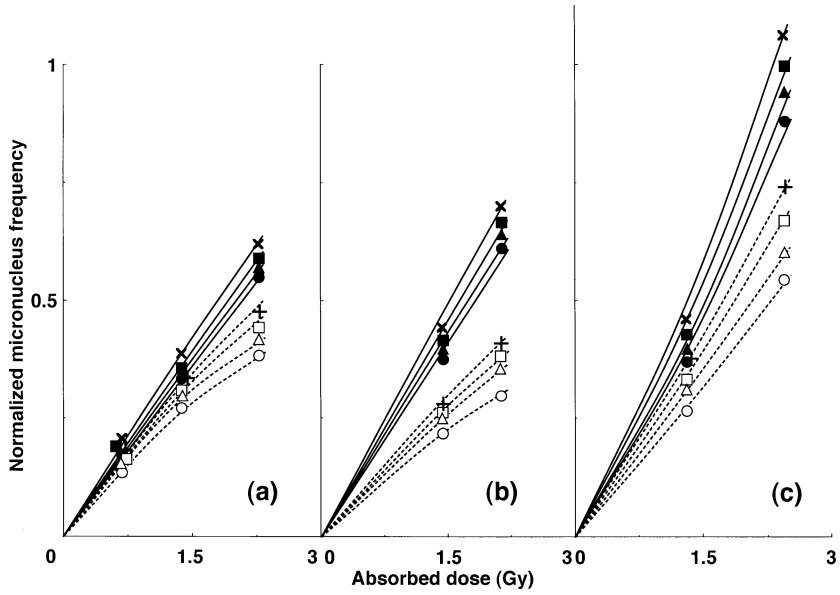

Fig. 4. Dose-response curves of normalized micronucleus frequency as a function of radiation dose for total (solid lines) and quiescent (dotted lines) tumor cell populations in EL4 tumors irradiated using reactor neutrons with BSH (a), BPA (b) or BPA-ol (c) in combination with MTH and/or TPZ treatment. ●, ■, ▲, × total cells; ○, □, △, + quiescent cells. ●, ○ without MTH or TPZ; ■, □ with TPZ without MTH; ▲, △ with MTH without TPZ; ×, + with both MTH and TPZ. MTH, mild temperature hyperthermia; TPZ, tirapazamine. Only mean values are shown, to avoid confusion.

cies increased in the following order; without MTH or TPZ<with MTH<with TPZ<with both MTH and TPZ. To evaluate the effect of MTH and/or TPZ on the frequencies in total and Q cell populations, the ER was calculated at various normalized frequencies using the values from the fitted curves shown in Figs. 4 through 6. ER was defined as the ratio of the absorbed radiation dose needed to obtain equivalent normalized frequencies without and with MTH and/or TPZ (Table IV). At each endpoint with any ^{10}B-compound for each cell population within both tumors, the value of ER increased in the following order; with MTH alone<with TPZ alone<with both MTH and TPZ. On the whole, the values of ER were larger for Q cells than for total cells. In total cells within both tumors, the values of

ER for both MTH and TPZ combined with BPA or BPA-ol were significantly larger than 1.00 ($P<0.05$). In Q cells within both tumors, the values for both MTH and TPZ combined with BSH and the values for TPZ alone and both MTH and TPZ combined with BPA or BPA-ol were significantly larger than 1.00 ($P<0.05$).

Further, to evaluate the effect of combined treatment with MTH and/or TPZ on the difference in sensitivity

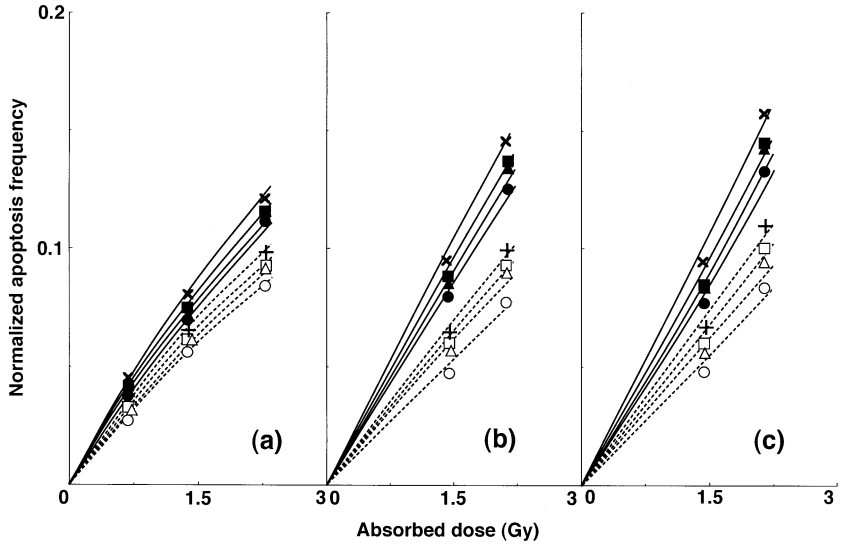

Fig. 5. Dose-response curves of normalized apoptosis frequency as a function of radiation dose for total (solid lines) and quiescent (dotted lines) tumor cell populations in EL4 tumors irradiated using reactor neutrons with BSH (a), BPA (b) or BPA-ol (c) in combination with MTH and/or TPZ treatment. Legends are the same as in Fig. 4. Only mean values are shown, to avoid confusion.

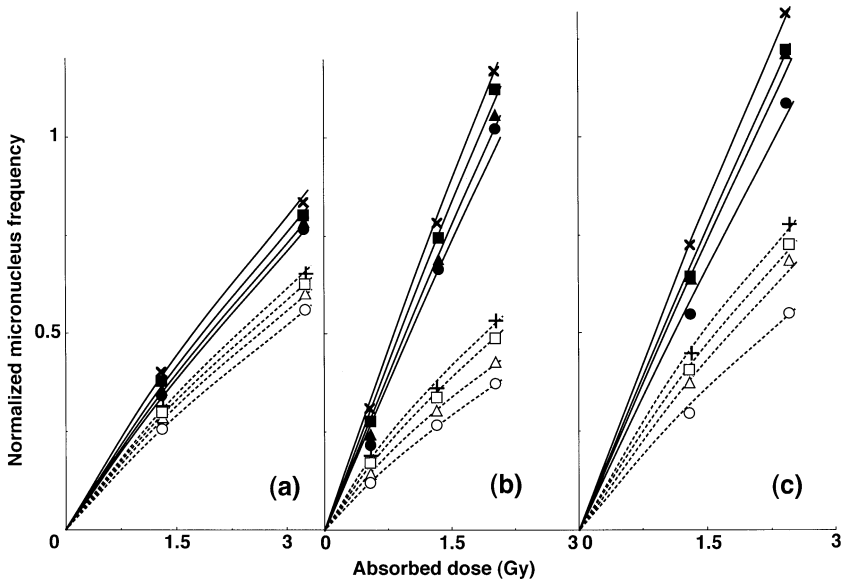

Fig. 6. Dose-response curves of normalized micronucleus frequency as a function of radiation dose for total (solid lines) and quiescent (dotted lines) tumor cell populations in SCC VII tumors irradiated using reactor neutrons with BSH (a), BPA (b) or BPA-ol (c) in combination with MTH and/or TPZ treatment. Legends are the same as in Fig. 4. Only mean values are shown, to avoid confusion.

Table IV. Enhancement Ratios[a] Due to TPZ[b] and/or Heating

Tumor	End point	Heating	TPZ	Heating+ TPZ
<Normalized micronucleus frequency>				
EL4				
Total cells				
With BSH	0.5	1.04	1.10	1.18
	0.2	1.03	1.08	1.17
With BPA	0.5	1.05	1.11	1.20
	0.2	1.05	1.12	1.20
With BPA-ol	0.5	1.04	1.13	1.21
	0.2	1.06	1.16	1.25
Quiescent cells				
With BSH	0.3	1.09	1.19	1.26
	0.2	1.09	1.17	1.25
With BPA	0.3	1.19	1.21	1.31
	0.2	1.18	1.27	1.37
With BPA-ol	0.5	1.11	1.21	1.31
	0.2	1.12	1.20	1.28
SCC VII				
Total cells				
With BSH	0.75	1.02	1.07	1.13
	0.3	1.02	1.07	1.17
With BPA	0.75	1.05	1.14	1.21
	0.3	1.07	1.15	1.24
With BPA-ol	0.75	1.12	1.16	1.26
	0.3	1.11	1.18	1.27
Quiescent cells				
With BSH	0.5	1.09	1.15	1.22
	0.2	1.07	1.13	1.21
With BPA	0.3	1.18	1.42	1.55
	0.2	1.17	1.39	1.51
With BPA-ol	0.5	1.26	1.37	1.50
	0.2	1.23	1.34	1.48
<Normalized apoptosis frequency>				
EL4				
Total cells				
With BSH	0.1	1.03	1.09	1.17
	0.04	1.04	1.12	1.19
With BPA	0.1	1.05	1.12	1.22
	0.04	1.05	1.14	1.22
With BPA-ol	0.1	1.05	1.10	1.21
	0.04	1.03	1.09	1.20
Quiescent cells				
With BSH	0.06	1.05	1.13	1.20
	0.04	1.05	1.14	1.21
With BPA	0.06	1.14	1.22	1.30
	0.04	1.13	1.22	1.29
With BPA-ol	0.06	1.12	1.22	1.32
	0.04	1.11	1.22	1.32

a) The ratio of radiation dose needed to obtain each end point without, and with, heating and/or tirapazamine treatment.
b) Tirapazamine.

between total and Q cell populations, we calculated the DMFs of Q cells relative to total cells using the values from the fitted curves shown in Figs. 4 through 6 (Table V). Combined treatment with TPZ alone or both MTH and TPZ significantly decreased the DMFs of Q cells compared with no combined treatment ($P<0.05$), only when either BPA or BPA-ol was used as a ^{10}B-compound.

Changes in the ^{10}B concentration in EL4 and SCC VII tumors on combined treatment with MTH and/or TPZ treatment (Fig. 7) show that only MTH could increase the uptake of ^{10}B-compound into both tumor cells, although not significantly, and that the combination with TPZ had essentially no effect on the uptake potential of tumor cells.

DISCUSSION

As shown in our previous report,[10] whether a conventional TUNEL (deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling) method or the fluorescence staining method employed here was used, in both tumor cells, the apoptosis frequency showed a similar profile with a maximum at 6 h after irradiation. Thus, as far as these two tumor cells are concerned, the fluorescence staining method was thought to reflect apoptotic events as well as the conventional TUNEL method. Meanwhile, the contribution of apoptosis to determining the response of tumors depends on the cell type[9] as well as the induction of MN after DNA-damaging treatment.[21] Namely, the ratio of apoptosis or MN formation to total cell death depends on the cell type.[9, 10] As also reported in our previous study,[10, 18] the time course of change in the value of the apoptosis frequency showed that death by apoptosis was well related to a response to γ-ray or reactor neutron radiation in EL4 tumors, but not in SCC VII tumors. That is to say, the ratio of apoptosis to total cell death is relatively high in EL4 tumor cells, but not in SCC VII tumor cells. In contrast, total cell death preferentially depends on MN formation in SCC VII tumor cells. Therefore, the induced normalized MN frequencies had relatively smaller values as a whole in EL4 tumor cells than in SCC VII tumor cells (Figs. 2 and 3). In addition, as also demonstrated in our previous report,[10] since correlations between apoptosis frequency and surviving fraction (SF) and between MN frequency and SF in total cells of both tumors had significant linearity ($P<0.001$), both apoptosis frequency and MN frequency reflect the sensitivity of these two tumor cells.

Actually, in both total and Q cells of EL4 tumors, similar curve patterns for all conditions could be obtained, based on the data of the MN frequency or apoptosis frequency (Figs. 2, 4 and 5). This indicates that the apoptosis frequency, as well as the MN frequency, reflects the sensitivity to neutron beams of EL4 tumor cells. Additionally, it is also shown that the apoptosis frequency, as well as the

Table V. The Effects of Heating and/or Tirapazamine on the Dose Modifying Factor for Quiescent Tumor Cells Relative to Total Tumor Cells[a)]

Tumor	End point	Heating (–) TPZ[b)] (–)	Heating (+) TPZ (–)	Heating (–) TPZ (+)	Heating (+) TPZ (+)
<Normalized micronucleus frequency>					
EL4					
With BSH	0.3	1.18	1.23	1.20	1.22
	0.2	1.18	1.19	1.15	1.17
With BPA	0.3	1.93	1.74	1.68	1.67
	0.2	1.85	1.72	1.63	1.63
With BPA-ol	0.5	1.78	1.55	1.33	1.32
	0.2	1.69	1.47	1.30	1.28
SCC VII					
With BSH	0.5	1.51	1.37	1.32	1.31
	0.2	1.47	1.41	1.30	1.28
With BPA	0.3	2.47	2.24	2.01	1.98
	0.2	2.40	2.13	1.81	1.79
With BPA-ol	0.5	2.47	1.90	1.65	1.63
	0.2	2.44	1.89	1.46	1.45
<Normalized apoptosis frequency>					
EL4					
With BSH	0.06	1.26	1.25	1.24	1.23
	0.04	1.23	1.22	1.22	1.21
With BPA	0.06	1.72	1.64	1.50	1.48
	0.04	1.70	1.62	1.49	1.46
With BPA-ol	0.06	1.72	1.52	1.38	1.36
	0.04	1.70	1.49	1.36	1.34

a) The ratio of radiation dose required to obtain each end point in quiescent tumor cells in relation to the radiation dose required to obtain each end point in total tumor cells.
b) Tirapazamine.

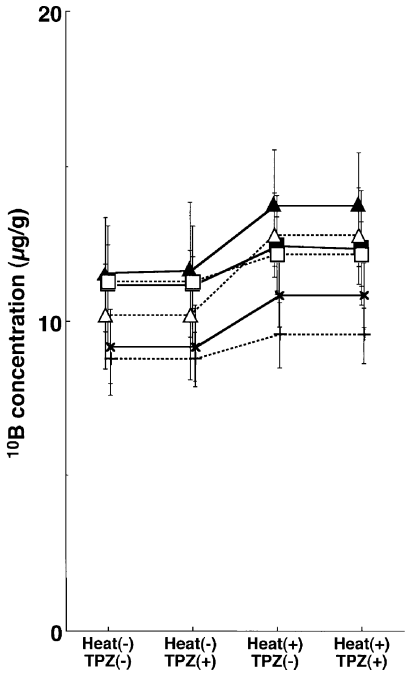

Fig. 7. Changes in the ^{10}B concentration in EL4 (solid lines) and SCC VII (dotted lines) tumors on combined treatment with MTH and/or TPZ. ■, ▲, × EL4 tumors; □, △, + SCC VII tumors. ■, □ BSH administration; ▲, △ BPA administration; ×, + BPA-ol administration. MTH, mild temperature hyperthermia; TPZ, tirapazamine. Bars represent 95% confidence limits.

MN frequency, can be applied to our method for measuring the Q cell response to reactor neutron beams within solid tumors in which the ratio of apoptosis to total cell death is relatively high, as in the EL4 tumor.

We have been investigating the radiobiological characteristics of two clinically used ^{10}B-carriers, BSH and BPA, especially from the viewpoint of their distribution in tumor cells.[18, 22] It is thought that the distribution of ^{10}B from BSH in tumor cells is more dependent on the diffusion of the drug than that from BPA, and that the distribution of ^{10}B from BPA is more dependent on the ^{10}B uptake ability of the tumor cells than that from BSH. Here, we estimated the radiobiological characteristics of the newly developed ^{10}B-carrier BPA-ol, which was designed and synthesized by Kirihata and his colleagues,[2, 3] comparing them with those of the two clinically used ^{10}B-carriers.

The use of a ^{10}B-compound increased the sensitivity of both total and Q cell populations, especially that of total cells (Table II), resulting in a widening of the difference in sensitivity between total and Q cells (Table III). This is probably because Q cells cannot take up ^{10}B as efficiently as P cells. In addition, in both tumors, the sensitivity of Q cells treated with BPA was lower than that of the cells treated with BSH, although the average ^{10}B concentration after BPA administration was almost the same as that after BSH in the total tumor cells. In other words, when BPA was used, ^{10}B was distributed to the total tumor cells as effectively as when BSH was used. However, less ^{10}B was localized into Q cells than when BSH was administered. These findings supported the putative mechanism of ^{10}B distribution from the two clinically used ^{10}B-carriers, BSH and BPA, into tumor cells.

On the other hand, BPA-ol significantly increased the sensitivity of total cells even compared with BPA ($P<0.05$), and, like BSH, significantly raised the sensitivity of Q cells compared with BPA ($P<0.05$). However, the sensitivity difference between total and Q cells was as large as with BPA and significantly larger than with BSH ($P<0.05$), since the degree of increase in the sensitivity of total cells was significantly larger than that of Q cells ($P<0.05$), as in the case of BPA. Since the dependency on ^{10}B uptake ability of the tumor cells was little changed, almost the same ^{10}B distribution pattern was obtained in the solid tumor as in the case of the parent drug, BPA, resulting in much less ^{10}B being distributed into Q cells than P cells. However, the highly water-soluble character of BPA-ol might cause an increase in the affinity for DNA molecules of tumor cells, which are in general electrically charged, although the ^{10}B concentration in the tumor cell as a whole was almost the same as or a little lower than that in the case of BPA. In other words, even with a similar or slightly lower ^{10}B concentration in the tumor cells as a whole, when BPA-ol was employed, a greater amount of ^{10}B might be distributed closer to the DNA molecules within the tumor cells (due to higher affinity) than when BPA was used. As a result, BPA-ol may have greater potential to cause DNA damage through neutron capture reaction.

When BPA or BPA-ol was used as a ^{10}B-carrier, combined treatment with TPZ significantly enhanced the sensitivity of Q cells ($P<0.05$), and combined treatment with both MTH and TPZ significantly enhanced the sensitivity of both total and Q cells ($P<0.05$) with a larger enhancement ratio for Q cells (Table IV). As a result, combined treatment with TPZ and both MTH and TPZ significantly reduced the sensitivity difference between total and Q cells, compared with the treatment without MTH or TPZ ($P<0.05$), when BPA or BPA-ol was used as a ^{10}B-carrier (Table V). According to our previous reports, Q tumor cell populations have much larger hypoxic fractions than P cells and are comprised of large chronically hypoxic, that is, oxygen diffusion-limited, hypoxic fractions,[23] and MTH has the potential to oxygenate chronically hypoxic fractions through an increase in tumor blood flow in heated tumors.[24] Thus, a hypoxic cell cytotoxin, TPZ, could kill Q tumor cells selectively and efficiently because of its hypoxic cell-specific cytotoxicity toward hypoxic cell-rich Q tumor cell populations. MTH could also enhance the sensitivity of chronic hypoxia-rich Q cell fractions, resulting from the distribution of higher doses of ^{10}B through an increase in tumor blood flow. Actually, as shown in Fig. 7, MTH increased ^{10}B uptake into both tumor cells, although not significantly, but TPZ had no substantial effect on the ^{10}B uptake. In BNCT, once enough ^{10}B for the neutron capture reaction is distributed in tumor cells, ^{10}B itself can enhance the sensitivity very efficiently, whether in combination with MTH and/or TPZ or not, because of the generation of heavily charged high LET and high RBE particles by the neutron capture reaction. Therefore, combination with TPZ and/or MTH could show larger ERs for Q cells than for total cells, especially when BPA or BPA-ol, which showed a poorer distribution in Q cells than BSH, was administered (Table IV). Accordingly, combined treatment with MTH and/or TPZ is thought to be effective in terms of sensitizing Q cells, to which it is difficult to deliver ^{10}B after the administration of a ^{10}B-carrier such as BPA or BPA-ol. Combined treatment with both MTH and TPZ seems very promising in terms of sensitizing both total and Q cells and reducing the sensitivity difference between total and Q cells, according to the outcome of the combination study of MTH and/or TPZ.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells.[4] The presence of these cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumor core, and this is another consequence of poor vascular supply.[4] This might promote MN formation and apoptosis in Q tumor cells, partly

due to the effect of hypoxia-induced apoptosis (Table I).[25] It has been reported that Q cells have lower radiosensitivity than P cells in solid tumors *in vivo*.[4, 6, 23] This means that more Q cells can survive after anticancer treatment than P cells. Consequently, the control of Q cells has a great impact on the outcome of anticancer therapy. From the viewpoint of the tumor cell killing effect including intratumor Q cell control, the newly developed ^{10}B-carrier BPA-ol has considerable potential, especially when administered with both MTH and TPZ. In the future, we intend to examine the radiobiological characteristics of an enantiomer of BPA-ol, that is, *d-p*-boronophenylalaninol-^{10}B, which can be synthesized from the parent drug *d-p*-boronophenylalanine-^{10}B through the same synthetic route as BPA-ol. Further, we also plan to investigate the response of intratumor Q cells to reactor neutron beams with other isotopes for the neutron capture reaction with thermal neutrons, such as gadolinium-157 (^{157}Gd), using our method for determining the sensitivity of Q tumor cell populations in solid tumors to various anticancer treatments.

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