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Effect of Electroporation on Cell Killing by Boron Neutron Capture Therapy Using Borocaptate Sodium (^{10}B-BSH)

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The cell membrane permeability of ^{10}B-enriched borocaptate sodium (BSH) and the extent to which BSH is accumulated in cells are controversial. To elucidate these points and to enhance the accumulation of BSH in cells, the effect of electroporation on boron neutron capture therapy (BNCT) using BSH was investigated. The first group of SCCVII tumor cells was incubated in culture medium with ^{10}B-BSH or ^{10}B-enriched boric acid, and exposed to neutrons from the heavy water facility of the Kyoto University Reactor. More than 99% of neutrons were thermal neutrons at flux base. The second group was pretreated with electroporation in combination with ^{10}B-BSH, and thereafter the cells were irradiated with neutrons. The cell-killing effect of BNCT was measured by colony formation assay. The surviving cell fraction decreased exponentially with neutron fluence, and addition of BSH significantly enhanced the cell-killing effect of NCT depending on ^{10}B concentration and the preincubation time of cells in the BSH-containing culture medium. The electroporation of cells with BSH markedly enhanced the BNCT effect in comparison with that obtained with preincubation alone. The effect of BSH-BNCT with electroporation was almost equal to that of BNCT using ^{10}B-boric acid at the same ^{10}B concentration. The effect of BNCT on cells pretreated with BSH and electroporation was not reduced by repeated washing of the cells before neutron irradiation. Decrease of the effect of BSH-BNCT plus electroporation with increase in the waiting time between the electroporation and the neutron irradiation could be explained in terms of the extent of cell growth during that time. These data suggest that BSH penetrates the cells slowly and remains after washing. Electroporation can introduce BSH into the cells very efficiently, and BSH thus introduced stays in the cells and is not lost in spite of the intensive washing of the cells. Therefore, if electroporation is applied to tumors after BSH injection, ^{10}B would remain in the tumors but be cleared from normal tissues, and selective accumulation of ^{10}B in tumors will be achieved after an appropriate waiting time.

Key words: BNCT — Electroporation — Borocaptate sodium

^{10}B absorbs thermal neutrons at an extremely high probability {3837 barn (cm^2)} in comparison with ^{1}H, ^{12}C, ^{14}N and ^{16}O, and emits an α particle and a recoiling ^{7}Li ion with an average total kinetic energy of 2.34 MeV through ^{10}B(n, α)^{7}Li reaction. These particles are high linear energy transfer radiation, and their tracks do not exceed one cell diameter (10 μm).[1)] Therefore, if ^{10}B could be accumulated selectively in tumor cells, the cells could be destroyed completely with minimal effects on adjacent cells containing no ^{10}B. This nuclear reaction has been applied to the treatment of malignant glioma and malignant melanoma.[2)] ^{10}B-enriched borocaptate sodium (BSH: $Na_2B_{12}H_{11}SH$) is an agent for boron neutron capture therapy (BNCT) of malignant glioma.[3)] BSH does not cross the blood brain barrier (BBB) into the normal brain, but accumulates in malignant brain tumors because of their disrupted BBB. However, the membrane permeability of BSH and BSH accumulation in tumor cells remain controversial. Moreover, in tumors in other organs, BSH distribution is not selective because of the lack of an appropriate selective barrier such as the BBB.[2)] Because the tracks of α particles and recoiling ^{7}Li ions are very short, the energy deposition in DNA varies depending upon the site of the boron neutron capture reaction. Biological effectiveness decreases with the distance between the reaction site and the cell nucleus, and this difference in cell killing can be easily detected by colony formation assay. If a method could be found to inject BSH into cells, the above controversy could be resolved by comparing the cell-killing effects of BSH-BNCT with and without this method. It has been reported that passage of an electric current across a cell membrane can increase its permeability, and this technique (known as electroporation) has frequently been applied to introduce drugs into cells.[4)] In this study, electroporation was employed to introduce BSH into cells, and its availability as an approach for improving BSH-BNCT was investigated.

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MATERIALS AND METHODS

Cell line and BSH The murine squamous cell carcinoma SCCVII was used in these studies. Tumor cells, exponentially growing in Eagle's minimum essential medium supplemented with 292 mg/liter *l*-glutamine and 12.5% fetal calf serum, were trypsinized and single cell suspensions in complete medium were prepared. BSH was purchased from BBI (Boron Biologicals, Inc., Raleigh, NC). It was dissolved in saline at about 400 ppm (as ^{10}B base), and the solution was sterilized by filtration. The ^{10}B concentration of the BSH solution was precisely measured by prompt-γ-ray spectrometry using a thermal neutron guide tube installed at Kyoto University Reactor (KUR).[5] The BSH solution was added to tumor cell suspensions containing $50-100\times10^4$ cells per ml at final ^{10}B-concentrations of 10 and 20 ppm.

Electroporation Tumor cell suspensions containing BSH were put into the chamber, maximum effective volume of 0.8 ml, of a Bio-Rad Laboratories Gene Pulser for electroporation. Electroporation was performed under the following conditions: electrical field strength=0.75 kV/cm (actual voltage=0.3 kV/0.4 cm electrode gap), capacitance=960 μF. Treatment time ranged from 10 to 12 s. The electroporation induced disruption, detected under a phase-contrast microscope, in about 80% of the cells.

Neutron irradiation After electroporation, the cell suspension was centrifuged, resuspended in fresh complete medium with or without BSH, and 2 ml of cell suspension was put into a small Teflon tube for thermal neutron irradiation. As a control experiment, the tumor cells were incubated for 2 or 16 h in the medium containing BSH and thereafter the suspension was centrifuged, resuspended in fresh complete medium containing or not containing BSH, and exposed to thermal neutron irradiation. To examine the ability of electroporation to allow BSH to enter cells, the cells suspended in complete medium containing ^{10}B-enriched boric acid were irradiated with neutrons, and the effect of BSH-BNCT plus electroporation with that of BNCT using boric acid was compared by colony formation assay. The ^{10}B concentration of ^{10}B-enriched boric acid was measured by prompt-γ-ray spectrometry, as was done for BSH solution. A neutron beam with a cadmium ratio of 148, i.e., a mixed beam of 2×10^9 thermal, 1.4×10^7 epithermal and 2.8×10^6 fast neutrons at flux base ($cm^{-2}s^{-1}$), was produced by the heavy water facility of KUR for the study. The neutron fluence was measured by the spectrometry of γ-rays from ^{198}Au produced in the reaction of ^{197}Au(n, γ)^{198}Au as reported previously.[6] Au foils were placed on both sides, front and back of the Teflon tube, and the fluence of neutrons that reached the cells was taken as the arithmetic mean value.

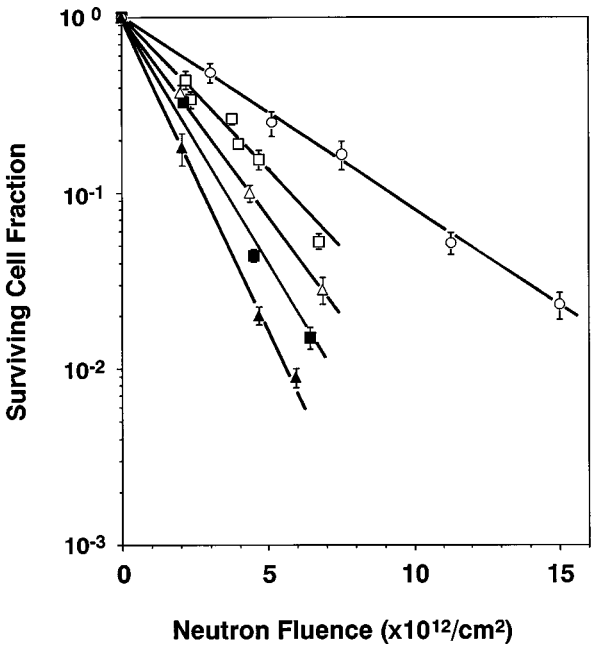

Fig. 1. Effect of ^{10}B concentration and preincubation time on cell survival curves following BNCT. □ BSH (^{10}B 10 ppm) 2-h incubation-rinse-neutrons, ■ BSH (^{10}B 10 ppm) 16-h incubation-rinse-neutrons, △ BSH (^{10}B 20 ppm) 2-h incubation-rinse-neutrons, ▲ BSH (^{10}B 20 ppm) 16-h incubation-rinse-neutrons, ○ neutron irradiation alone.

Table I. Parameters of Cell Survival Curves of SCCVII Tumor Cells following Various BSH-BNCT

Treatment group	$-\ln SF=C+\alpha\phi$	
	C	$\alpha\pm SD$ ($\times10^{-12}$)
1) neutrons alone	–0.0011	0.254±0.0105
2) ^{10}B 10 ppm (BSH), 2-h incubation, R+	–0.0608	0.427±0.0369
3) ^{10}B 10 ppm (BSH), 16-h incubation, R+	–0.0924	0.675±0.0735
4) ^{10}B 20 ppm (BSH), 2-h incubation, R+	–0.0198	0.525±0.0116
5) ^{10}B 20 ppm (BSH), 16-h incubation, R+	–0.0360	0.802±0.0496

ϕ: neutron fluence. R: rinse.

Colony formation assay After irradiation, an appropriate number of tumor cells to form 50 to 100 colonies was inoculated onto 60 mm diameter Petri dishes containing complete culture medium. After incubation for 7 days, the colonies were fixed with ethanol, stained with crystal violet, and counted under a low power microscope. The plating efficiency (PE) of the cells without electroporation or the cells that escaped destruction by electroporation was 84.5±6.8%. The surviving cell fraction (SF) was obtained by dividing the PE of the cells exposed to neutrons by that of cells not exposed to neutrons. The best-fitted relationship between neutron fluence and SF was determined by using the least-squares method.

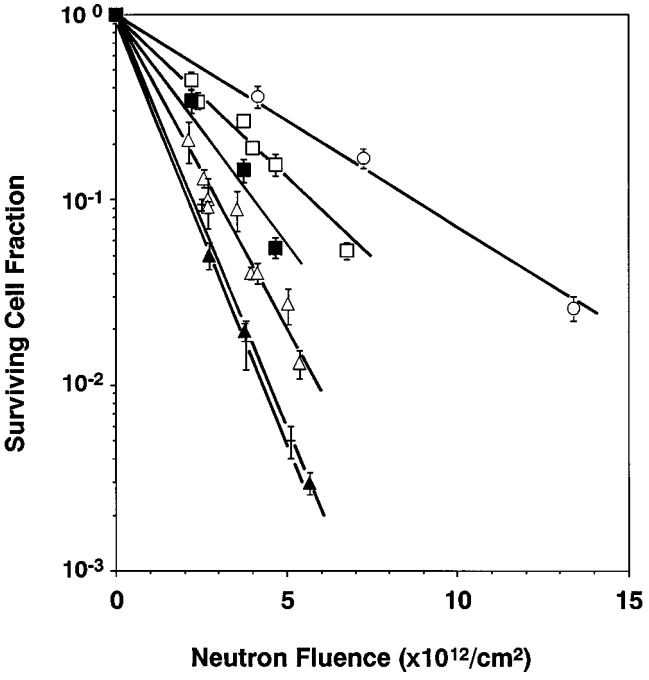

Fig. 2. Effect of electroporation on cell killing by NCT with or without boron compounds. □ BSH (^{10}B 10 ppm) 2-h incubation-rinse-neutrons, △ BSH (^{10}B 10 ppm) electroporation-rinse-neutrons, + ^{10}B-enriched boric acid (^{10}B 10 ppm)-neutrons, ○ electroporation-rinse-neutrons, ▲ BSH (^{10}B 10 ppm) electroporation-rinse(−)-neutrons, ■ BSH (^{10}B 10 ppm) 2-h incubation-rinse(−)-neutrons. The data of BSH (^{10}B 10 ppm) 2-h incubation-rinse-neutrons were taken from Fig. 1 for comparison.

RESULTS

The surviving cell fraction decreased exponentially with neutron fluence, and addition of BSH significantly enhanced the cell-killing effect of NCT in a ^{10}B concentration-dependent manner, i.e., the slopes of cell survival curves were $0.254(\pm0.0105)\times10^{-12}$ cm^{2}s, $0.427(\pm0.0369)\times10^{-12}$ cm^{2}s and $0.525(\pm0.0116)\times10^{-12}$ cm^{2}s for neutron irradiation alone, for 10 ppm ^{10}B 2-h incubation and for 20 ppm ^{10}B 2-h incubation, respectively (Fig. 1 and Table I). The effect of BNCT also significantly increased with preincubation time, i.e., the cell survival slopes for 16-h incubation were $0.675(\pm0.0735)\times10^{-12}$ cm^{2}s and $0.802(\pm0.0496)\times10^{-12}$ cm^{2}s at 10 ppm and 20 ppm ^{10}B, respectively (Fig. 1 and Table I).

The electroporation alone did not increase the sensitivity of cells to neutrons, i.e., the slope of the cell survival curve was $0.273(\pm0.0125)\times10^{-12}$ cm^{2}s for addition of electroporation without BSH (Fig. 2 and Table II). On the other hand, the electroporation of cells with 10 ppm ^{10}B BSH markedly enhanced the BSH-BNCT effect in comparison with that of simple preincubation with BSH. The slopes of the cell survival curves were $0.763(\pm0.060)\times10^{-12}$ cm^{2}s and $0.427(\pm0.0369)\times10^{-12}$ cm^{2}s for BSH plus electroporation and for BSH without electroporation, respectively (Fig. 2 and Table II). The effect of BSH-BNCT with electroporation was smaller than that of BNCT using ^{10}B-boric acid at the same ^{10}B concentration (10 ppm), i.e., the α value for boric acid is $1.053(\pm0.0382)\times10^{-12}$ cm^{2}s (Fig. 2 and Table II). In the boric

Table II. Changes of α Values of Cell Survival Curves of SCCVII Tumor Cells following BNCT with or without Electroporation and Rinsing of Cells

Treatment group	$-\ln SF=C+\alpha\phi$	
	$\alpha\pm$SD ($\times10^{-12}$)	$\Delta\alpha$ ($\times10^{-12}$)
6) EP×1, R+	0.273±0.0125	
2) ^{10}B 10 ppm (BSH), 2-h incubation, R+	0.427±0.0369	
7) ^{10}B 10 ppm (BSH), 2-h incubation, R−	0.598±0.0586	0.171 {7)−2)}
8) ^{10}B 10 ppm (BSH), EP×1, R+	0.763±0.0600	0.490 {8)−6)}
		0.336 {8)−2)}
9) ^{10}B 10 ppm (boric acid), R−	1.053±0.0382	
10) ^{10}B 10 ppm (BSH), EP×1, R−	1.027±0.0257	0.264 {10)−8)}

ϕ: neutron fluence. EP: electroporation. R: rinse.

acid experiment, ^{10}B-boric acid was not removed from the medium by centrifugation before neutron irradiation. When centrifugation was not applied to the cells treated with BSH plus electroporation, the cells showed almost equal sensitivity to neutrons as that of ^{10}B-boric acid BNCT. Centrifugation to remove BSH was repeated twice or three times after electroporation, and the change of sensitivity of the cells to neutrons was examined in order to evaluate the ability of BSH to remain in the cells. No reduction of the sensitivity was observed, i.e., the cell survival levels were not higher than those of the cells centrifuged once before neutron irradiation (Fig. 3).

In the above studies, electroporation for injecting BSH into the cells was performed only once. Therefore, in the next study the effect of repeated electroporation in the presence of BSH on the cell sensitivity to neutrons was investigated. As presented in Fig. 3, the neutron sensitivity of the cells was further enhanced in comparison with that of cells exposed to a single electroporation, i.e., the slopes of the cell survival curves were $0.788(\pm0.0541)\times10^{-12}$ cm^{2}s and $1.173(\pm0.0341)\times10^{-12}$ cm^{2}s for one and two electroporations, respectively (Fig. 3 and Table III).

The cells treated with BSH plus electroporation were kept in an incubator for 20 h before neutron irradiation to examine the extent of loss of BSH from the cells during this waiting time. The sensitivity of the cells to neutrons decreased, i.e., the slopes of the cell survival curve were $0.767(\pm0.0462)\times10^{-12}$ cm^{2}s and $0.449(\pm0.0271)\times10^{-12}$ cm^{2}s for neutron irradiation immediately after electroporation

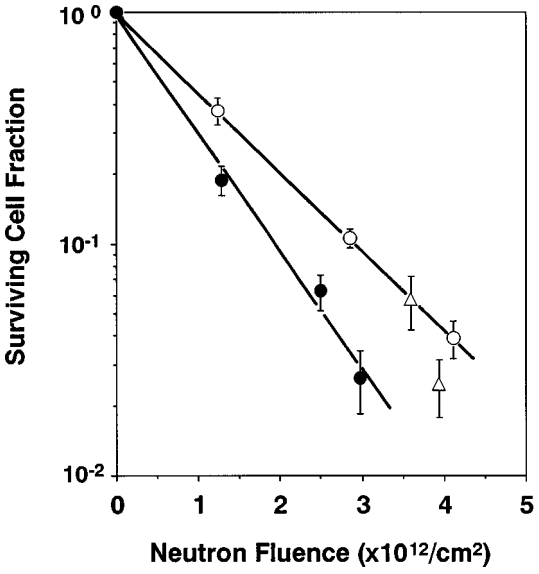

Fig. 3. Effect of repetition of electroporation and rinsing after electroporation on the cell killing by BNCT with BSH (^{10}B 10 ppm). ○ electroporation×1-rinse×1-neutrons, ● electroporation×2-rinse×1-neutrons, △ electroporation×1-rinse×2 or 3-neutrons.

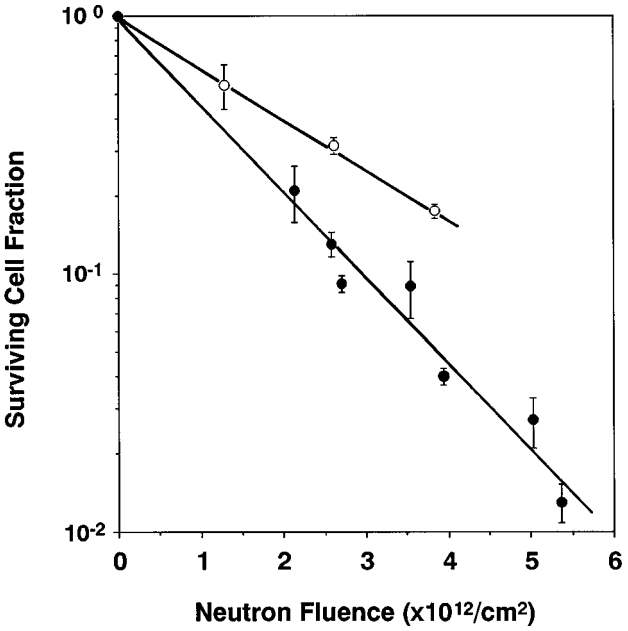

Fig. 4. Effect of waiting time until neutron irradiation after electroporation. ● BSH (^{10}B 10 ppm) electroporation-rinse-0 h-neutrons, ○ BSH (^{10}B 10 ppm) electroporation-rinse-20 h-neutrons.

Table III. Effects of Repetition of Electroporation or Waiting Time between Electroporation and Neutron Irradiation on α Values of Cell Survival Curves of SCCVII Tumor Cells following BNCT

Treatment group	$-\ln SF=C+\alpha\phi$	
	$\alpha\pm SD$ ($\times10^{-12}$)	$\Delta\alpha$ ($\times10^{-12}$)
6) EP, R+	0.273±0.0125	
11) ^{10}B 10 ppm (BSH), EP×1, R+	0.788±0.0541	0.515 {11)–6)}
12) ^{10}B 10 ppm (BSH), EP×2, R+	1.173±0.0341	0.385 {12)–11)}
13) ^{10}B 10 ppm (BSH), EP×1, R+	0.767±0.0462	0.494 {13)–6)}
14) ^{10}B 10 ppm (BSH), EP×1, R+, 20 h	0.449±0.0271	0.176 {14)–13)}

ϕ: neutron fluence. EP: electroporation. R: rinse.

and for neutron irradiation 20 h after electroporation, respectively (Fig. 4 and Table III).

DISCUSSION

As described in the introduction, the membrane permeability of BSH and BSH accumulation in tumor cells are still controversial. Some investigators argue that BSH accumulates in tumor cells at higher concentrations than in the blood, i.e., the tumor/blood ratio is 1.3–1.46.[7, 8] However, others found that the tumor/blood ratio of boron concentration does not exceed unity in clinical cases.[9, 10] Furthermore, almost complete loss of the BSH-BNCT effect was reported when cells were washed, even after 24-h preincubation.[11] Different results were obtained in our present study, i.e., the BNCT effect increased with preincubation time of cells with BSH and did not disappear after washing (Fig. 1 and Table I). These findings suggest that BSH slowly enters cells.

Electroporation alone before neutron irradiation did not alter the radiosensitivity of the cells in comparison with control cells (Fig. 1 and Table I vs. Fig. 2 and Table II). This finding implies that electroporation does not always destroy cells in the radiation-resistant phase of the cell cycle. When electroporation was applied to cells in combination with BSH, the neutron sensitivity of the cells was remarkably enhanced in comparison with that of control cells or cells given preincubation treatment alone in the BSH-containing medium (Fig. 2 and Table II). Even when centrifugation was repeated twice or three times to remove BSH after electroporation, no reduction of the sensitivity was observed, i.e., the cell survival levels were not higher than those of the cells centrifuged once (Fig. 3). These results mean that electroporation can efficiently inject BSH into the cells and BSH in the cells is not readily washed out. ^{10}B-boric acid is considered to enter the cells freely, i.e., intracellular ^{10}B concentration can reach the same level as in the medium, and it markedly enhanced the effect of NCT (Fig. 2 and Table II). On the other hand, the neutron sensitivity did not reach the level of the cells in the medium with ^{10}B-boric acid. However, the cells pretreated with BSH plus electroporation without rinsing showed an equivalent neutron sensitivity (Fig. 2 and Table II). Therefore, the difference of neutron sensitivity between the cells following BSH plus electroporation with rinsing and the cells in the medium containing ^{10}B-boric acid is attributable to the effect of ^{10}B existing outside the cells in the medium. The differences in the α values of cell survival curves which were observed in a comparison between cells with and without removal of BSH by centrifugation were smaller than the extent of increase in α value by addition of electroporation to BSH (Table II). This finding is consistent with the physical characteristics of α and ^{7}Li in the boron-10 neutron capture reaction, i.e., the tracks of particles which are emitted outside cells are too short efficiently to kill the cells.

When electroporation for injecting BSH into the cells was repeated twice, the neutron sensitivity of the cells was further enhanced in comparison with cells which received a single electroporation (Fig. 3 and Table III). Furthermore, the increase of the sensitivity was larger than that by ^{10}B-boric acid in the medium at the same ^{10}B concentration (Tables II and III). This indicates that repeated electroporation is very effective in injecting BSH into cells, although the reason why higher sensitivity was achieved in comparison with ^{10}B-boric acid is still speculative. BSH that entered cells might be trapped by covalent mixed disulfide bonding between glutathione and BSH.[12] Consequently, the BSH concentration would be lower than that outside the cells, and further entry of BSH might be allowed at the next electroporation depending on the concentration gradient. The GSH level in SCCVII tumors is 1.1 mmol/kg,[13] and is much higher than the BSH level in the medium, because BSH contains 12 ^{10}B atoms in a molecule and therefore 10 ppm BSH as ^{10}B base is equivalent to 0.0833 m*M* as BSH base.

The increase in neutron sensitivity of cells achieved by BSH plus electroporation decreased to 35.6% (0.176/0.494=0.356) during a waiting time of 20 h until neutron irradiation (Fig. 4 and Table III). The cell cycle time of SCCVII tumor cells is about 14 h and the number of cells increased by a factor of 2.7 in 20 h. Therefore if BSH does not leak from the cells after electroporation, BSH is diluted to 37% of the initial level. This agrees well with the observed decrease of neutron sensitivity. This finding suggests that BSH is very stable in cells.

The clinical implications of our data seem to be as follows. After administration of BSH to cancer patients, BSH clears from blood with a $T_{1/2}$ of 6–7 h.[10] Therefore, if electroporation is applied to tumors after BSH injection, ^{10}B should remain in tumors but be cleared from normal tissues, and selective accumulation of ^{10}B in tumors should be achieved after an appropriate waiting time. BSH may become applicable, as a BNCT agent, to various tumors other than malignant glioma in the brain. The enhancement of accumulation of BSH in tumors by electroporation has been reported in an experimental rat brain tumor model, although the neutron sensitivity has not been investigated.[14] We have just started a study on the neutron sensitivity of *in vivo* tumors pretreated with BSH plus electroporation. In a preliminary study, BSH was markedly accumulated in SCCVII tumors grown in C3H/He mice when electroporation was applied.

As an alternative to electroporation, shock waves might be effective to increase accumulation of BSH in tumors. When shock waves were applied, in combination with bleomycin, to adenocarcinoma transplanted in nude mice, enhanced drug toxicity was observed.[15] The cytotoxicity

of bleomycin against adenocarcinoma is quite low, and one of the causes of this resistance might be the cellular membrane blocking the entry of the drug into the cells. Shock waves can increase membrane permeability to bleomycin, as does electroporation.[16] Therefore, similar effects are anticipated for BSH. Moreover, shock waves can be focused on lesions situated deep in the body. We are planning to investigate the effect of BSH plus shock waves using SCCVII tumor cells *in vitro* and *in vivo*.

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