

いことがその主な原因ではあるが周囲に放射線感受性の高い小腸などが存在するので主病巣と広い腹部への根治線量の照射が一般に困難である。BPAは腸管上皮に良く集積するので利用できないが、将来、膵癌細胞に選択的に集積する硼素化合物が開発されれば、中性子捕捉療法は膵癌の有力な治療法の一つになる可能性がある。

#### 4. 新規ホウ素化合物の開発

機序から分かるようにBNCTの成否は、ホウ素化合物に負うところが大きい。1968年にBSH (Borocaptate Sodium)が<sup>13)</sup>、そして1987年にBPAが<sup>14)</sup>臨床研究に使われて各々40年以上、20年以上が過ぎたが患者に試用された新規のホウ素化合物は未だない。その理由は、化合物が十分に高い腫瘍選択性と高い集積濃度と言う同時には達成し難い特性を具備しなければならないからである。その為、開発努力が向けられた方向は全く新規の化合物の開発ではなく、BSHとBPAを出発物質とした化学修飾物の開発やDDSを利用した効率の良い腫瘍への配送に係る研究である。①BSH内封リポソームとその表面をPEGで標識しマクロファージによる貪食を回避しつつ Transferrin<sup>15)</sup>や抗EGFR抗体<sup>16)</sup>のような腫瘍で高発現しているreceptorに対する抗体で標識し選択的配送を狙う。②peptide (poly-Arg) をによる細胞への蛋白導入法の応用<sup>17)</sup>や陽イオン化gelatin HVJ-E<sup>18)</sup>での標識によってBSHに細胞膜透過性を与える。③リポソームの膜にホウ素を組み込んだ高集積化ホウ素リポソーム<sup>19)</sup>によって輸送効率を高める。④浸透圧利用による高濃度での局所投与。⑤低酸素細胞毒とホウ素化合物の複合体、等々、様々な試みが為されているが、ヒトへの試用には未だ距離がある。今後の研究の促進が必要である。

研究の促進の鍵となる技術は、ホウ素の分布をミクロのレベルで画像化する確実で簡便な手法であろう。如何なるホウ素化合物の分布検索にも応用できる手法の開発である。現行の $\alpha$ -オートラジオグラフィでは粒子の飛程が大きくホウ素原子の位置を細胞レベルの精度では特定できない。斯うした点を克服した画像化技術の開発は候補となるホウ素化合物の効果、とりわけ正常組織に対する生物効果を推定しホウ素化合物の新規開発を促進することに繋がる技術と考えている。

肝癌のBNCTで見られる様に臨床的技術によって腫瘍選択性を改善した効率的な薬剤の送達も直ちに応用可能な手法として重要である。選択的動脈注入、動脈塞栓術などTVR技術を用いた薬剤送達の工夫である。

#### 5. 医療承認

これまでBNCTは研究治療として実施されてきた。研究炉の実績から考えれば、医療としての公的承認を得なければならぬ段階に達していると考える。原子炉は大きなフラックスが得られる点で魅力的であるが、特有の規制や定期点検での長期に亘る運転休止期間が生まれる等、医療に本格的に用いるには致命的な欠点がある。その為、中性子源として原子炉を用いる限り、特別な規制緩和が無ければ原子炉BNCTの薬事承認を得ることは不可能である。幸いサイクロトロン中性子源が開発され、病院にも設置可能な仕様であることから、早期の薬事治験の開始を目指して規制当局との折衝や手続きが、現在、正に進行中である。薬事治験が終了しBNCTの公的承認が得られれば、臨床のみならず、関連分野の基礎研究をも急速に進むことになるであろう。

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## Biological Evaluation of Dodecaborate-Containing L-Amino Acids for Boron Neutron Capture Therapy

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**ABSTRACT:** To develop a boron carrier for practical purposes, new boron-containing amino acids with an undecahydro-*closo*-dodecaboranylthio ( $[\text{}^{10}\text{B}_{12}\text{H}_{11}\text{S}]^{2-}$ ) unit in the side chain of the  $\alpha$ -amino acid have already been designed and synthesized. In the present paper, cytotoxicity, the incorporation amounts into tumor cells, and the tumor cell killing effects of these compounds were elucidated to evaluate their usefulness as boron carriers. Furthermore, the microdistribution of the amino acids in tumor cells was established.

## INTRODUCTION

Recently, boron neutron capture therapy (BNCT) has become recognized as an essential therapy for brain cancer, head and neck cancer, and melanoma.<sup>1</sup> BNCT is based on the nuclear capture and fission reactions of the  $^{10}\text{B}$  atom with low energy thermal/epithermal neutrons to yield high linear energy transfer  $\alpha$  particles and recoiling  $^7\text{Li}$  nuclei. Because the path lengths of the particles are approximately 9–10  $\mu\text{m}$ , equal to the dimensions of a single cell,  $^{10}\text{B}$ -containing cells are selectively destroyed by BNCT.

Although many kinds of boron compounds, including amino acids, nucleic acids, and liposomes, have been reported as boron delivery agents (boron carriers) for BNCT,<sup>2–6</sup> only two compounds, *p*-borono-*L*-phenylalanine (BPA) and disodium mercapto-*closo*-undecahydrododecaborate ( $[\text{B}_{12}\text{H}_{11}\text{SH}]^{2-} 2\text{Na}^+$ , BSH), are clinically used in treatment of cancer with BNCT.<sup>7</sup> For a boron delivery agent to be successful in BNCT, the compound must have following properties: (i) high tumor targeting selectivity ( $T/N > 3\text{--}4:1$ ), (ii) low systemic toxicity, (iii) concentrations of  $\sim 20 \mu\text{g } ^{10}\text{B}/\text{g}$  tumor tissues, especially the boron compound, is preferred to accumulate into tumor cell and/or cell nuclei, and (iv) high water-solubility. On the other hand, the *L*-amino acid transport system in tumor cells is enhanced to ensure cell multiplication compared with normal tissues. Therefore, there has been a long-standing interest in the design, synthesis, and biological evaluation of boron-containing  $\alpha$ -amino acids with tumor-seeking and tumor-localizing properties.

*L*-BPA has been used as an excellent boron delivery agent for BNCT. Because *L*-BPA is selectively uptaken into tumor cells, particularly accumulating in the cell nuclei, *L*-BPA is clinically used in BNCT.

BSH and its derivatives are of increasing interest as boron carriers for BNCT with the aim to deliver large amounts of  $^{10}\text{B}$

atoms to tumor cells.<sup>8</sup> BSH is a class of water-soluble boron cluster compound and has low toxicity compared with another boron cluster compounds. Owing to these properties, BSH is clinically used for the treatment of brain tumors with BNCT as a  $^{10}\text{B}$  carrier,<sup>9</sup> although tumor selectivity of BSH is slightly low.

To develop practical materials utilizing  $^{10}\text{B}$  carriers, we previously designed and synthesized several new boron-containing amino acids, including C4-BSH-AA ( $n = 2$ ) (**1a**), C5-BSH-AA ( $n = 3$ ) (**1b**), and C8-BSH-AA ( $n = 6$ ) (**1c**), which include the undecahydro-*closo*-dodecaboranylthio ( $[\text{}^{10}\text{B}_{12}\text{H}_{11}\text{S}]^{2-}$ ) unit through a boron–sulfur–carbon bond connection to the side chain of  $\alpha$ -amino acid<sup>10</sup> (Figure 1).

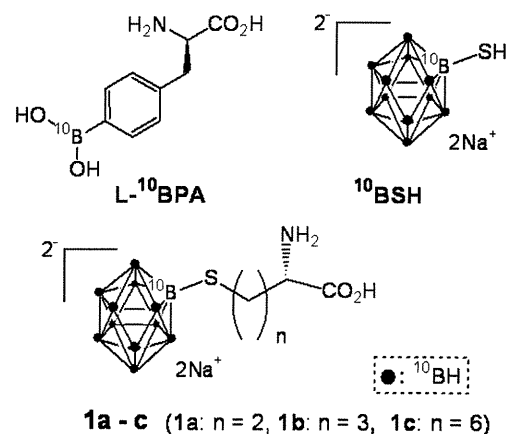
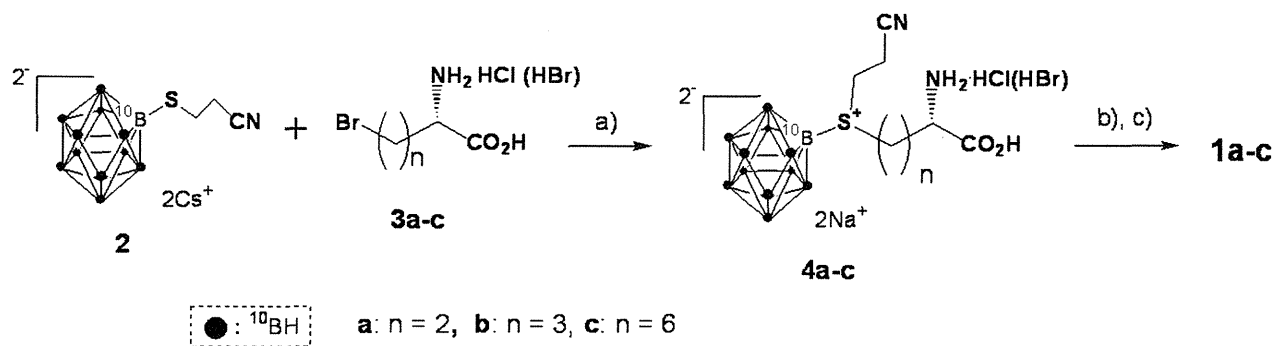


Figure 1. Boron-containing compounds.

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Scheme 1. Synthesis of BSH-Amino Acids (1a–c)<sup>a</sup>

<sup>a</sup>Reagents and conditions: (a) MeCN, reflux, 24 h; (b) Me<sub>4</sub>NOH, MeNH<sub>2</sub>, acetone, rt, 30 min; (c) amberlite IR-120(Na<sup>+</sup>).

Here, we report the distribution of the BSH-amino acids 1a–c in tumor cells and their cytotoxicities, the incorporated amount into cancer cells, and the tumor cell killing effects.

## MATERIALS AND METHODS

**General.** L-<sup>10</sup>BPA and <sup>10</sup>BSH were provided by Stella Pharma Corporation (Osaka, Japan). Capillary electrophoresis analysis was carried out using Photal CAPI-3300 (Otsuka Electronics Co. Ltd., Osaka, Japan) equipped with a fused silica capillary of 75 μm i.d. and a total length of 50 cm (effective length of 38 cm). A new capillary was conditioned with 0.1 M NaOH for 30 min followed by 15 min of distilled water and 15 min of electrolyte buffer (5 mM sodium phosphate buffer, pH 9.0). The applied potential was 20 kV and detection at 200 nm. Inductively coupled plasma optical emission spectrometry (ICP-OES) was obtained on a VISTA-MPX ICP-OES spectrometer (Seiko Instruments, Chiba, Japan). Fluor 488 goat anti-mouse IgG was purchased from Life Technologies (Carlsbad, CA, US). Permafluor was purchased from Immunotech (Marseille, France).

**Synthesis of BSH-Amino Acids (1a, 1b, and 1c).** The synthesis of BSH-amino acids 1a–c was carried out according to previously described method.<sup>10</sup> The purity of 1a–c was analyzed by capillary electrophoresis, and the analysis of 1a–c was revealed the purity to be >95%.

**Cells and Cell Culture.** SAS (human oral squamous cell carcinoma), B16 (mouse melanoma), and C6 (rat glioma) cell lines, used in cytotoxicity analyses, boron incorporation, tumor cell killing effect, and immunostaining, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 24 mM sodium hydrogen carbonate at 37 °C in a 5% CO<sub>2</sub> atmosphere. Cells in the monolayer were harvested with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in Ca<sup>2+</sup>-free phosphate-buffered saline (PBS). Matrigel (growth factor reduced type) was purchased from BD Science (San Jose, CA, US).

**Water-Soluble Tetrazolium (WST)-8 Assay.** The cytotoxicity of each amino acid was assayed using the WST-8 assay with a cell counting kit (Wako Pure Chemicals, Osaka, Japan) following the manufacturer's protocols with some modifications. Briefly, a 96-well microplate was seeded with 5 × 10<sup>3</sup> cells suspended in 100 μL of cell growth media and allowed to settle for 16 h at 37 °C. The medium was removed by aspiration, and 100 μL of growth medium (DMEM with 10% FBS containing each compound at different concentrations) was added to each well. After incubation for 48 h at 37 °C, the medium was removed by aspiration and 100 μL of WST-8 solution [5 mM WST-8, 20 mM HEPES (pH 7.4) and 0.2 mM 1-methoxy PMS dissolved in PBS] were added to each well, followed by incubation for 4 h at 37 °C. The absorbance at 450 nm (reference: 655 nm) was read on a microwell plate reader. A well without cells was used as the blank. Relative cell survival was calculated as follows;

Table 1. Cytotoxicity of Boron Amino Acids against Tumor Cells

compd	IC <sub>50</sub> (mM)		
	C6	SAS	B16
L- <sup>10</sup> BPA	>2	>2	>2
C4-BSH-AA (1)	6.6	7.8	5.6
C5-BSH-AA (2)	2.4	4.9	6.6
C8-BSH-AA (3)	4.7	5.6	5.1

relative cell survival (%)

$$= \frac{\text{(absorbance value of compound treated well)}}{\text{absorbance value of untreated well}} \times 100$$

The relative cell survival was plotted against the compound concentration, and IC<sub>50</sub> values for each compound were graphically determined.

**Boron Incorporation into Cultured Tumor Cells.** Cultures were inoculated with 1.0 × 10<sup>6</sup> cells/dish, and cells were grown for 24 h in DMEM. The medium was replaced with an equivalent medium containing each boron-containing amino acid (the final concentration was 2.0 mM in each case). The cells were cultured for 24 h, and the medium was then removed by aspiration. The cells were washed thrice with PBS, harvested by trypsinization, and then counted. Each sample, containing 1 × 10<sup>6</sup> cells, was added to a mixture of HClO<sub>4</sub> (60%, 0.3 mL) and H<sub>2</sub>O<sub>2</sub> (31%, 0.6 mL) and then heated at 75 °C for 1 h. The mixture was filtered through a membrane filter (Millipore, 0.45 μm), and the boron concentration was measured by ICP-OES.

**Immunostaining of C6 Cells.** Immunostaining was performed to determine the incorporation of boron-containing amino acids into C6 cells, according a previously described method with some modifications.<sup>11</sup> Glass coverslips coated with Matrigel (3.5 μg/cm<sup>2</sup> protein) were seeded with C6 cells (0.8 × 10<sup>5</sup> cells suspended in 3 mL of DMEM) and allowed to settle for 1 h at 37 °C. The medium was replaced with an equivalent medium containing compound 1a (the final concentration was 2.0 mM in each case), and the cells were cultured for 24 h at 37 °C. After being washed with DMEM, C6 cells were fixed with 10% paraformaldehyde in PBS for 10 min at room temperature. The cells were rinsed with PBS and treated with 0.05% Triton X-100 for 10 min at room temperature. Further, the cells were washed with PBS and preincubated in a humid chamber with 1.0% BSA/0.02% NaN<sub>3</sub> in PBS at room temperature, followed by incubation with the anti-BSH monoclonal antibody A9H3 in PBS containing 1.0% BSA/0.02% NaN<sub>3</sub> (0.2 μg/mL) for 60 min at 32 °C. The cells were rinsed with PBS and then incubated with Alexa-Fluor 488 goat anti-mouse IgG in PBS containing 1.0% BSA/0.02% NaN<sub>3</sub> (0.2 μg/mL) for 30 min at 32 °C. After washing with PBS, the cells were mounted with Permafluor and then photographed with a microscope (IX-70, Olympus, Tokyo) equipped with a cooled charge-coupled device

camera (UIC-QE, Molecular Devices Co., Sunnyvale, CA, U.S.) controlled by MetaMorph software (Molecular Devices Co.).

**Tumor Cells Killing Effect Study.** Cultures were inoculated with  $1.0 \times 10^6$  cells/dish, and cells were grown for 24 h in DMEM. The medium was replaced with an equivalent medium containing each boron-containing amino acid (the final concentration was 2.0 mM in each case). They were cultured for 24 h, and the medium was removed by aspiration. The cells were washed with PBS, harvested by trypsinization, and then counted. After centrifugation, trypsin was removed by aspiration, and DMEM was added to the residual cells. The cell suspension in DMEM ( $5.0 \times 10^3$  cells/mL, 1 mL) was irradiated with thermal neutrons for 0–90 min in a column-shape tube. The thermal neutron fluence was determined by averaging two gold foils, symmetrically attached to the surface of the column-shape tube along the direction of incidence of the thermal neutrons. After thermal neutron exposure, 600 cells were placed in three Corning 60 mm tissue culture dishes containing 3 mL of DMEM to examine colony formation. Seven days later, the colonies were fixed with ethanol and stained with 0.1% crystal violet for quantitative visualization by the naked eye.

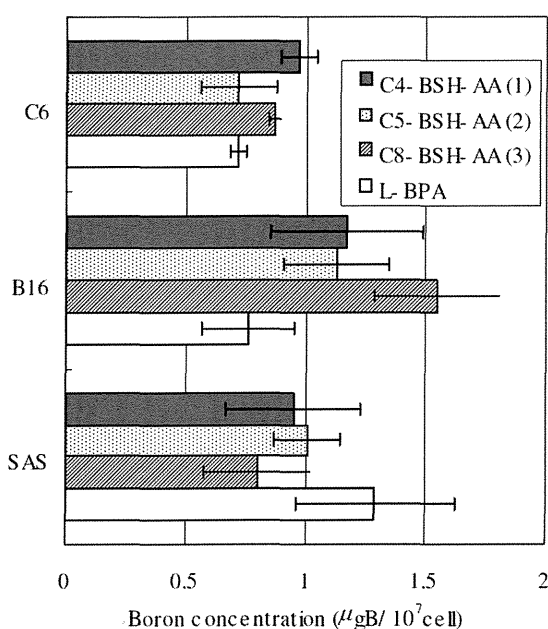


Figure 2. Incorporated amount of boron amino acids.

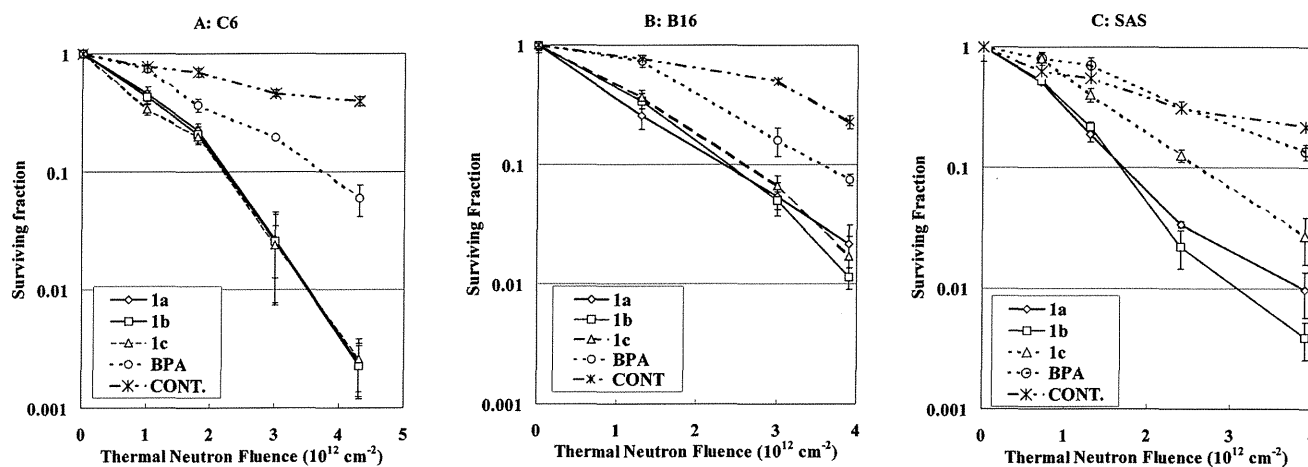


Figure 3. The tumor cell killing effects of boron-containing amino acids (A) against C6 cells, (B) against B16 cells, and (C) against SAS cells.

## RESULTS AND DISCUSSION

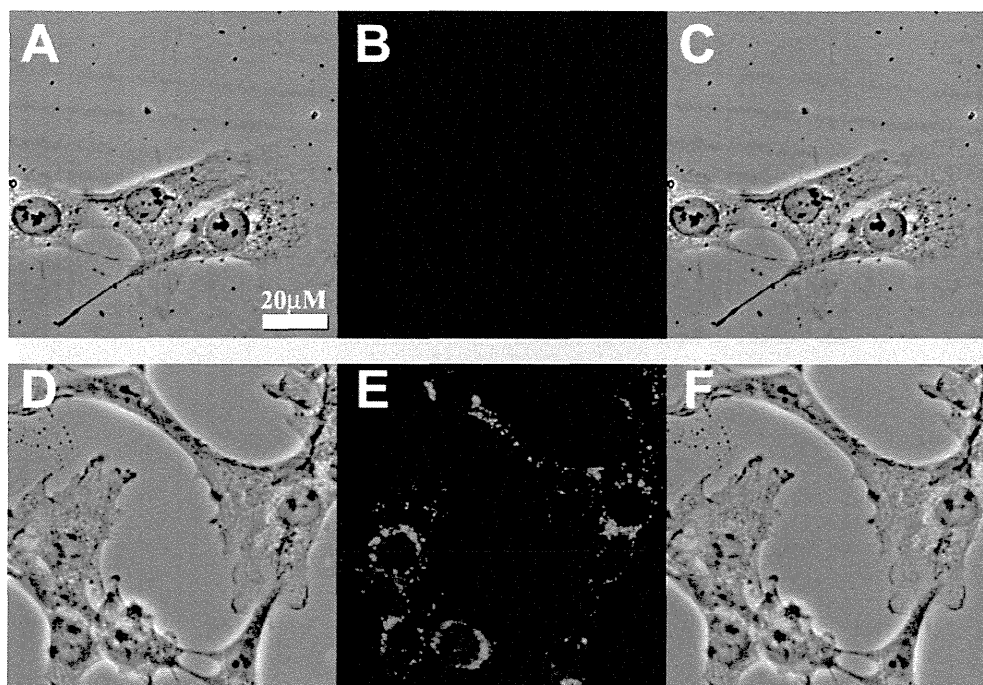
An alternative route of synthesis of the three BSH-amino acids (1a–c) is illustrated in Scheme 1. As shown, the starting compound, *S*-(cyanoethyl)-BSH tetramethylammonium salt (2),<sup>12</sup> was prepared with a high yield by hetero-Michael reaction of BSH dicesium salt with acrylonitrile, using sodium hydroxide as a base.<sup>13</sup> On the other hand, the  $\omega$ -bromo-*L*-amino acids (3a–c), represented as Br-(CH<sub>2</sub>)<sub>*n*</sub>-CH(NH<sub>2</sub>)-COOH (*n* = 2, 3, and 6, respectively), were prepared as hydrochloric or hydrobromic salts. Among these, (*S*)-2-amino-4-bromobutyric acid (3a, *n* = 2) was commercially purchased, and the others (3b and 3c) bearing the (*S*)-configuration were synthesized according to modified versions of published methods.<sup>14,15</sup>

The alkylation reaction of 2 with  $\omega$ -bromo-*L*-amino acids (3) was completed by a simple procedure. The purity and chemical structure of 1 were analyzed by <sup>1</sup>H and <sup>13</sup>C NMRs, ESI-MS, and capillary electrophoresis. In the present synthesis, absolute configuration of the starting  $\omega$ -bromo-*L*-amino acid was introduced to the final amino acids in retention without any racemization.

The cytotoxicities of the BSH-amino acids 1a–c and *L*-BPA toward the C6, SAS, and B16 cell lines were determined using the WST-8 test (Table 1). As shown in Table 1, the cytotoxicity of each compound was very low. However, the cytotoxicity of the three BSH-amino acids was higher than that of *L*-BPA.

To elucidate the incorporation amounts of BSH-amino acids 1a–c into cancer cells, we measured the boron concentrations in three kinds of cancer cells, C6, B16, and SAS by ICP-OES (Figure 1). The compounds 1a, 1b, and 1c were up-taken into cancer cells. In particular, the amounts of 1a–c that were incorporated were higher than those of *L*-<sup>10</sup>BPA for C6 and B16 cells. These results suggest that the BSH-amino acids 1a–c are as useful as *L*-<sup>10</sup>BPA as <sup>10</sup>B carrier.

To determine the distribution of each BSH-amino acid in tumor cells, we stained C6 cells that incorporated compound 1a with the anti-BSH antibody A9H3<sup>16</sup> (Figure 2). The results showed the distribution of BSH-amino acid 1a was very different from that of *L*-BPA. *L*-BPA was reported that widely distributed in the cytoplasm and the cell nuclei there are no regions within the cells in which the concentration of *L*-BPA is especially high.<sup>17,18</sup> In contrast, the compound 1a was



**Figure 4.** The microdistribution of compound **1a** in C6 cells. (A) A phase-contrast micrograph of C6 cells that were cultured in DMEM. (B) A fluorescence micrograph of C6 cells that were cultured in DMEM stained with the anti-BSH antibody A9H3. (C) A merged image of A and B. (D) A phase-contrast micrograph of C6 cells that were cultured in DMEM containing compound **1a**. (E) A fluorescence micrograph of C6 cells that were cultured in DMEM containing compound **1a** stained with the anti-BSH antibody A9H3. (F) Merged image of D and E.

incorporated into the cell membrane of the C6 cells and aggregated on the fringe of the cell nuclei (Figure 4).

To confirm the usefulness of the BSH-amino acids **1a–c** for BNCT, we examined the tumor cell killing effects of  $L$ - $^{10}\text{BPA}$  and compounds **1a–c** against tumor cells *in vitro* using neutron irradiation (Figure 3). Despite the finding that the amounts of the three amino acids incorporated were closely equivalent to that of  $L$ - $^{10}\text{BPA}$ , **1a–c** showed higher killing effects than  $L$ - $^{10}\text{BPA}$  for all types of tumor cells tested.

## CONCLUSION

From these results presented, we conclude that the undecahydro-*closo*-dodecaboranylthio unit is well suited as a boron source, and the BSH-amino acids **1a–c** are useful as  $^{10}\text{B}$  carriers because they aggregated on the fringe of the cell nuclei and showed higher cell-killing effects than  $L$ -BPA toward several tumor cells types. *In vivo* evaluation of compounds **1a–c** is ongoing, and the results will be reported soon.

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

BNCT, boron neutron capture therapy;  $L$ - $^{10}\text{BPA}$ ,  $p$ -( $^{10}\text{B}$ )-borono- $L$ -phenylalanine;  $^{10}\text{BSH}$ , mercapto-undeca-hydro-*closo*-dodeca( $^{10}\text{B}$ )borate; ICP-OES, inductively coupled plasma optical emission spectrometry; WST, water-soluble tetrazolium; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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