Antibody-Based Receptor Targeting Using an Fc-Binding Peptide-Dodecaborate Conjugate and Macropinocytosis Induction for Boron Neutron Capture Therapy

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ABSTRACT: Boron neutron capture therapy (BNCT) is a radiation method used for cancer therapy. Cellular uptake of boron-10 (10B) atoms induces cancer cell death by the generation of alpha particles and recoiling lithium-7 (7Li) nuclei when the cells are irradiated with low-energy thermal neutrons. Current BNCT technology shows effective therapeutic benefits in refractory cancers such as brain tumors and head and neck cancers. However, improvements to cancer targeting and the cellular uptake efficacy of the boron compounds and the expansion of the diseases treatable by BNCT are highly desirable. In this research, we aimed to develop an antibody-based drug delivery method for BNCT through the use of the Z33 peptide, which shows specific recognition of and interaction with the Fc domain of human IgG, for on-demand receptor targeting. In addition, we determined with an in vitro assay that macropinocytosis induction during antibody-based drug delivery is crucial for the biological activity of BNCT.

INTRODUCTION

Boron neutron capture therapy (BNCT) is nuclear capture-based radiotherapy. In BNCT, 10B (nonradioactive) compounds, including arylboronic acids [(l)-4-dihydroxyborylephynylanine, BPA] and polyhedral borane anion (disodium mercaptoundecahydro-closo-dodecaborate, BSH), which are currently being tested in clinical trials, are used because they generate α particles (4He) and recoiling 7Li nuclei in the range of 5−9 μm, which corresponds to the diameter of a cell, when irradiated by thermal neutrons (energy less than 0.5 eV) in the cells that internalize them, leading to cancer cell-killing activity.1−7 BNCT has the ability to effectively treat malignant brain cancer and head and neck cancer;7,8 however, its use as a cancer treatment is limited because of its particularly low cancer cell targeting efficacy, its poor cellular uptake efficiency, and the poor retention of boron compounds inside cancer cells.7,8 To attain effective BNCT biological activity, ~20 μg/g tumor weight of 10B must be selectively taken up by cancer cells (~10⁹ atoms/cell), and enough neutrons must be absorbed to sustain a lethal 10B(ν, α)/7Li capture reaction.9 Additional important still requirements for BNCT include low intrinsic toxicity and little uptake by normal tissues at tumor/normal tissue and tumor/blood boron concentration ratios of >3:1 in tailor-made therapy.8,9 In recent years, BNCT technology has been proceeded including, for example, block copolymer boron conjugate for the EPR effect for enhanced tumor accumulation.11 However, further technical improvements are greatly needed for future BNCT-based cancer therapy.

Antibody-based cancer receptor targets and intracellular delivery of therapeutic molecules are strong criteria for effective cancer treatment. Preparation of an antibody-therapeutic molecule conjugate without reducing the efficacy of antibody recognition is very important for generating a sophisticated cancer targeting system. The Z33 peptide, which is derived from the B-domain of protein A, has been shown to efficiently bind to an Fc domain of human IgG, for on-demand receptor targeting. In addition, we determined with an in vitro assay that macropinocytosis induction during antibody-based drug delivery is crucial for the biological activity of BNCT.

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which it can be applied (Figure 1). In addition, enhanced cellular uptake of the antibody-boron conjugates by effective macropinocytosis induction was shown to be important to increase the biological activity in BNCT after accumulation of boron compounds on plasma membrane using a receptor-target antibody (Figure 1).

### RESULTS AND DISCUSSION

Figure 1 shows the experimental concept of “cassette”-like binding of dodecaborate to an antibody receptor target on a cancer cell. Dodecaborate is conjugated to the Z33 peptide (Z33-DB), which binds to an Fc of the objective antibody. After Z33-DB is produced, the objective antibody is mixed with Z33-DB to easily form the “cassette” complex, which recognizes and binds to target receptors on the cancer membrane. On the other hand, cancer cells expressing high levels of macropinocytosis-inducing receptors, such as epidermal growth factor receptor (EGFR) and the CXCR4 chemokine receptor, can induce macropinocytic cellular uptake pathways by receptor activation. Macropinocytotic pathways are accompanied by clathrin-independent and actin-dependent plasma membrane ruffling and engulfment of large volumes (>1 μm) of extracellular fluids. For example, high expression of oncogenic K-Ras in pancreatic adenocarcinoma cells induces macropinocytosis to increase the cellular uptake of nutrition from outside cells, leading to malignant progression, and treatment of these cells with the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl)amiloride, which is not an anticancer reagent, reduced their proliferation in vivo. Next, EGFR receptor activation by EGF induces macropinocytosis, leading to efficient cellular uptake of Z33-DB. As described below in detail, only receptor targeting by an antibody specifically localizes to the therapy-relevant molecules on plasma membranes, and the active induction of macropinocytosis and efficient cellular uptake is needed to induce therapeutic biological activities. After cellular uptake, thermal neutron irradiation induces effective cancer cell-killing activity through BNCT (Figure 1).

Figure 2 shows the preparation methods for the conjugate of the Z33 peptide and dodecaborate. The Z33 peptide was synthesized by Fmoc-solid-phase synthesis methods, and the sulphydryl group of Cys in the Z33 peptide sequence was conjugated with BMOE (Z33-BMOE). Dodecaborate (BSH) was then subjected to another Michael addition reaction with Z33-BMOE, leading to the Z33-dodecaborate conjugate (Figure 2). The Z33-DB compound was
used for the “cassette” binding of the objective antibody and the receptor target in BNCT.

Before assessment of the Z33-DB-based receptor targets, we examined receptor recognition of the fluorescently labeled Z33 peptide (Z33-Alexa660) (Figure S1). In the experiments, A431 cells (derived from human epidermoid carcinoma), which express high levels of human EGFR on the plasma membrane, and CHO-K1 cells (derived from Chinese hamster ovary), which do not express human EGFR, were used for determining the recognition and accumulation of the Z33-Alexa660 that is in complex with the anti-EGFR antibody, cetuximab. Figure S1 shows the confocal laser microscopic observation of the A431 cells and CHO-K1 cells treated with Z33-Alexa660 (200 nM) for 30 min at 4 °C to prevent endocytosis. According to our results, the complex formed with cetuximab (100 nM) greatly enhanced the accumulation of Z33-Alexa660 on the plasma membranes of the A431 cells (Figure S1). In the case of the CHO-K1 cells, the Z33-Alexa660 and cetuximab complex did not accumulate on the plasma membrane, suggesting that the Z33-Alexa660/cetuximab complex specifically targeted the objective receptor (Figure S1). We also synthesized a fluorescently labeled random amino acid sequence of the Z33 peptide (rZ33-Alexa660) and tested whether it accumulated on the plasma membrane. The randomized Z33 peptide sequence did not accumulate on the A431 cells even with the addition of cetuximab (Figure S1).

Next, we evaluated the effects of EGF cotreatment in inducing the cellular uptake of the Z33-Alexa660/FITC-labeled cetuximab (FITC-cetuximab) complex through macro-pinocytosis. EGFR activation by treatment with the receptor ligand EGF induced signal transduction that initiates macro-pinocytosis via the activation of Rac (Rho family of small GTPase), which leads to actin organization and lamellipodia formation.16,17 First, we assessed macro-pinocytosis induction through treatment with EGF, and we found that the cellular uptake of the macro-pinocytosis marker FITC-labeled dextran (70 kDa)22,23 was significantly increased by treatment with EGF, as analyzed using confocal laser microscopy and flow cytometry under experimental conditions (Figures S2 and S3). Enhanced macro-pinocytotic cellular uptake was confirmed at >12 h after the EGF co-treatment (Figure S2). Therefore, our experimental condition of cellular Z33-Alexa660/FITC-cetuximab complex uptake was fixed as 24 h treatment. Figures 3a, S4, and S5 show confocal laser microscopy captured images of the A431 cells treated with the Z33-Alexa660 (200 nM)/FITC-cetuximab (100 nM) complex for 24 h at 37 °C, and cotreatment with EGF (100 nM) substantially increased the cellular uptake of the Z33-Alexa660/FITC-cetuximab complex compared to the uptake by cells not cotreated with EGF. In addition, the random amino acid sequence of the Z33 peptide did not increase the cellular uptake, even after the EGF was used to stimulate macro-pinocytosis induction (Figure 3a). In addition, we confirmed the induction of macro-pinocytosis by EGF treatment even in the presence of cetuximab under experimental conditions using a macro-pinocytosis marker (Figure S3). Figure 3b also shows the results from the flow cytometry analysis of Z33-Alexa660 binding to the A431 cell membrane and the cellular uptake based on the l-ethylendiaminetetraacetic acid disodium salt solution (EDTA) methods, as described in the Experimental Section. Specifically, the complex consisting of Z33-Alexa660 and cetuximab with and without EGF was shown to have significantly enhanced membrane binding ability. These results suggest that EGF treatment did not affect the binding efficacy of the Z33 peptide/cetuximab complex to the plasma membranes and that treatment with EGF increased the cellular uptake of the Z33 peptide/cetuximab complex bound to the plasma membrane. Trypsinization is very difficult to completely detach antibody from targeted receptors and plasma membrane. However, we conducted flow cytometry analysis in the methods of trypsinization, as described in the Experimental Section, and EGF treatment increased cellular fluorescent intensity of Z33-Alexa660/cetuximab (Figure S6). In addition, EGF treatment also increased cellular fluorescent intensity of Z33-Alexa660 without cetuximab. However, increased cellular uptake level of Z33-Alexa660 by EGF treatment was lower than that of the Z33-Alexa660/cetuximab complex without EGF treatment. In this in vitro assay, co-treatment of the Z33-Alexa660/cetuximab complex was focused on because in flow circumstances in body, Z33 peptides without the antibody might not be accumulated on targeted tumor cells and might be eliminated. Therefore, in this research, we focus on further experiments of cellular uptake of boron compounds and thermal neutron irradiation using the Z33 peptides/cetuximab complex and EGF in vitro assay.
In addition, in our experiments, we adopted and used cetuxumab anti-EGFR antibody, which is an antagonist and blocks the activation of EGFR. Therefore, binding of cetuximab to the targeted EGFR blocks the receptor activation and cellular uptake of the cetuximab-bound EGFR by clathrin-mediated endocytosis. However, EGF activates the EGFR without binding of cetuximab on the plasma membrane leading to induction of macropinocytosis, and then, the cetuximab-bound EGFR might be taken up by cells by macropinocytosis, which can induce membrane ruffling, nonspecific engulfment, and cellular uptake.

We next assessed the cellular receptor recognition and cellular uptake of Z33-DB. Figures 4a and S7 show confocal laser microscopic images of the A431 cells treated with Z33-DB (each 200 nM) with or without cetuximab (cet) (100 nM) and/or EGF (100 nM) in cell culture medium containing 10% FBS for 24 h at 37 °C. Before being observed, the cells were fixed with 4% PFA and 0.1% Triton-X 100 and then stained with an anti-BSH antibody [first antibody: A9H3 anti-BSH-Mab, second antibody: Alexa-Fluor 488 goat anti-mouse IgG(H + L)]. Green: Z33-DB. Scale bar: 20 μm. Enlarged pictures (areas within the white dotted square) are shown in Figure S7. (b) ELISA assay for detecting boron concentration in 1.0 × 10^7 cells of A431 treated with Z33-DB (each 200 nM) with or without cetuximab (cet) (100 nM) and/or EGF (100 nM) in cell culture medium containing 10% FBS for 24 h at 37 °C. The data are expressed as the mean (±SD) of three experiments.

Figure 4. (a) Confocal laser microscopic images of the A431 cells treated with Z33-DB (each 200 nM) with or without cetuximab (cet) (100 nM) and/or EGF (100 nM) in cell culture medium containing 10% FBS for 24 h at 37 °C. Before being observed, the cells were fixed with 4% PFA and 0.1% Triton-X 100 and then stained with an anti-BSH antibody [first antibody: A9H3 anti-BSH-Mab, second antibody: Alexa-Fluor 488 goat anti-mouse IgG(H + L)]. Green: Z33-DB. Scale bar: 20 μm. Enlarged pictures (areas within the white dotted square) are shown in Figure S7. (b) ELISA assay for detecting boron concentration in 1.0 × 10^7 cells of A431 treated with Z33-DB (each 200 nM) with or without cetuximab (cet) (100 nM) and/or EGF (100 nM) in cell culture medium containing 10% FBS for 24 h at 37 °C. The data are expressed as the mean (±SD) of three experiments.

In this research, we developed a "cassette-like" antibody binding technique for the receptor-targeted delivery of boron compounds based on BNCT. Active induction of macropinocytosis was crucial for the efficient cellular uptake of the boron compounds in targeted cancer cells and their biological activity after thermal neutron irradiation. Prof. Barth et al. pointed out that development of BNCT technology for delivery of boron compounds to targeted tumor cells on the

CONCLUSIONS

In this research, we developed a "cassette"-like antibody binding technique for the receptor-targeted delivery of boron compounds based on BNCT. Active induction of macropinocytosis was crucial for the efficient cellular uptake of the boron compounds in targeted cancer cells and their biological activity after thermal neutron irradiation.
bonds of e.g., porphyrins, polyamines, nucleosides, liposomes nanoparticles, however, the best way to further improve the clinical efficacy of BNCT would be to optimize the dosing paradigms and delivery of boron compounds. Of course, cell-targeted delivery is one of the very important techniques to specifically deliver the objective boron compounds into tumor cells in BNCT to prevent damaging on normal cells and inducing side effects. As active targeting to objective tumor cells, antibodies against tumor receptors have been applied and conjugated to each carrier molecule with boron compounds. As shown in our research results, anti-EGFR antibody can recognize and accumulate on the plasma membrane via EGFR; however, only accumulation of the antibodies on the plasma membranes can be observed without effective cellular uptake of the antibody-conjugated carrier molecules with boron compounds. In our experiments, even in vitro assessments, only accumulation of boron compounds on the plasma membrane shows low cancer-killing activity after thermal neutron irradiation. Enhanced cellular uptake of the antibody-boron conjugates by effective macrophocytosis induction might be needed to induce the sophisticated biological activity in BNCT. The “cassette-like” antibody binding system to boron compounds is considered to be very useful for development of the tailor-made tumor target system with very easy handling. The system is considered to be possibility to attain future tumor targeting on demand. Our main focus in this research is the analysis and evaluation of the delivery mechanism of the Z33-antibody complex system, and the experiments on the boron carrier can be interpreted as an example of a demonstration experiment for the proof of concept with being applicable for improving previous approaches using various boron compound-carrier techniques. Our findings contribute not only to the development of the intracellular delivery of boron compounds in BNCT but also to an understanding of the importance of the effective cellular uptake of therapeutic molecules in the strategy of cancer receptor targeting.

**EXPERIMENTAL SECTION**

**Peptide Synthesis.** All peptides were chemically synthesized via the 9-fluorenlymethoxy carbonyl (Fmoc) solid-phase synthesis method on a Rink amide resin with coupling reagents of 1-hydroxybenzotriazole (HOBt)/2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Peptide Institute, Osaka, Japan)/N-methylmorpholine (NMM) (Nacalai Tesque, Kyoto, Japan) in dimethylformamide (DMF) (Nacalai Tesque) as previously described. The Rink amide resin and the Fmoc-amino acid derivatives were purchased from Shimadzu Biotech (Kyoto, Japan) and the Peptide Institute (Osaka, Japan), respectively. Deprotection of the protected peptide and cleavage from the resin were conducted via treatment with a trifluoroacetic acid/ethanediol mixture (95:5) for 3 h at 25 °C, followed by reverse-phase HPLC purification. The purification of each peptide was estimated to be >97% on the basis of the analytical HPLC peaks. The structures of the synthesized peptides were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Microflex, Bruker, Billerica, MA, USA).

Z33-GGC (NH2-Phe-Asn-Met-Gln-Gln-Gln-Arg-Arg-Phe-Tyr-Glu-Ala-Leu-His-Asp-Pro-Asn-Leu-Glu-Glu-Gln-Arg-Asn-Ala-Lys-Ile-Lys-Ser-Ile-Arg-Asp-Asp-Gly-Gly-Cys-amide): MALDI-TOFMS: 4321.4 [calcd. for (M + H)+: 4321.7]. Retention time in HPLC, 11.0 min (column: Cosmosil SC18-AR-II (4.6 × 150 mm); gradient: 5–95% B in a (A = H2O containing 0.1% CF3COOH; B = CH3CN containing 0.1% CF3COOH) over 30 min; flow: 1 mL/min; detection 220 nm). The yield from the starting resin was 2.3%.

Z33-GGC (NH2-Phe-Asn-Met-Gln-Gln-Arg-Arg-Phe-Leu-Asn-Phe-Ala-Leu-Asn-Arg-Pro-Asn-Leu-Glu-Glu-Gln-Arg-Asn-Ala-Lys-Ile-Lys-Ser-Ile-Arg-Asp-Asp-Gly-Gly-Cys-amide): MALDI-TOFMS: 4321.5 [calcd. for (M + H)+: 4321.7]. Retention time in HPLC, 11.0 min (column: Cosmosil SC18-AR-II (4.6 × 150 mm); gradient: 5–95% B in a (A = H2O containing 0.1% CF3COOH; B = CH3CN containing 0.1% CF3COOH) over 30 min; flow: 1 mL/min; detection 220 nm). The yield from the starting resin was 0.2%.

For the preparation of fluorescently labeled peptides, synthesized and purified peptides were subjected to reactions with Alexa Fluor 660 C6 maleimide (Thermo Fisher Scientific Inc., Rockford, IL, USA) in the presence of NMM (0.5% v/v) in methanol/DMF (1:1) for 3 h at 25 °C and then purified by HPLC. The purity of each peptide was estimated to be >97% on the basis of the analytical HPLC results. The structures of the synthesized peptides were confirmed using MALDI-TOFMS.

**Figure 5.** (a) Surviving fraction of the A431 cells treated with or without BSH (200 nM), Z33-DB (200 nM), cetuximab (100 nM), and EGF (100 nM) for 24 h at 37 °C, prior to thermal neutron irradiation (90 min), and a colony assay, as described in the Experimental Section. The data are expressed as the mean (±SD) of three experiments. (b) Surviving fraction of A431 cells in the same experimental conditions (thermal neutron fluence 3.7 × 107). The data are expressed as the mean (±SD) of three experiments. *P < 0.05, **P < 0.01.
Z33-GGC-Alexa660 [(NH2-Phe-Asn-Met-Gln-Gln-Gln-Arg-Arg-Phe-Tyr-Glu-Ala-Leu-His-Asp-Pro-Gly-Gly-Cys (Alexa 660)-amide)]: MALDI-TOFMS: 5453.1 [calcld. for (M + H)+: 5453.8] (Figure S9)]. Retention time in HPLC: 13.5 min (column: Cosmosil SC18-AR-II (4.6 × 150 mm)); gradient: 5–95% B in A (A = H2O containing 0.1% CF3COOH; B = CH3CN containing 0.1% CF3COOH) over 30 min; flow: 1 mL/min; detection 220 nm). The yield from the starting resin was 0.03%.

**Preparation of the Dodecaborate-Z33 Peptide Conjugate.** For the preparation of the disodium mercaptopoundecahydrododecoborate (BSH)-Z33 peptide conjugate, synthesized and purified Z33-GGC peptide (1 equiv) was first subjected to reaction with bismaleimide ethane (BMOE) (3 equiv) in the presence of NMM (0.05% v/v) in methanol/DMF (1:1) for 6 h at 25 °C and then was purified by HPLC purification. The BMOE-linked Z33 peptide was then subjected to react with 10BSH [provided by Stella Pharma Corporation (Osaka, Japan)] in methanol/DMF (1:1) for 1 h at 25 °C (Figure S10) and then was purified by HPLC purification. The purity of each peptide was estimated to be >97% on the basis of the analytical HPLC results. The structures of the synthesized peptides were confirmed using MALDI-TOFMS.

**Z33-BMOE [(NH2-Phe-Asn-Met-Gln-Gln-Gln-Gln-Arg-Arg-Phe-Tyr-Glu-Ala-Leu-His-Asp-Pro-Asn-Leu-Asn-Glu-Glu-Arg-Asn-Ala-Lys-Ile-Lys-Ser-Ile-Arg-Asp-Glu-Gly-Cys (BMOE)-amide)]: MALDI-TOFMS: 5453.1 [calcld. for (M + H)+: 5452.8] (Figure S9)]. Retention time in HPLC: 11.1 min (column: Cosmosil SC18-AR-II (4.6 × 150 mm)); gradient: 5–95% B in A (A = H2O containing 0.1% CF3COOH; B = CH3CN containing 0.1% CF3COOH) over 30 min; flow: 1 mL/min; detection 220 nm). The yield from the starting resin was 0.3%.

**Preparation of Fluorescently Labeled Cetuximab.** Cetuximab (Erbitux, Merck Biopharma Japan, Tokyo, Japan) was purified by washing with Dulbecco’s phosphate-buffered saline (PBS) (Nacalai Tesque) and filtration using Amicon Ultra centrifugal filters (100 K device, Merck Millipore, Billerica, MA, USA). The concentrations of cetuximab were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). Purified cetuximab in PBS was fluorescein labeled using fluorescein isothiocyanate isomer I (FITC) (Sigma-Aldrich, St. Louis, MO, USA) (1 equiv) in the presence of NMM (1 equiv) in PBS for 1 h at 25 °C, prior to filtration using Amicon Ultra centrifugal filters (100 K device, Merck Millipore). The antibody concentration of the FITC-labeled cetuximab was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.).

**Cell Cultures.** Human epidermoid carcinoma-derived A431 cells and Chinese hamster ovary (CHO)-K1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Each cell line was cultured in MEM ( Gibco, Life Technologies Corporation, Grand Island, NY, USA) for A431 cells and F-12 nutrient mixture (Ham’s F-12) for CHO-K1 cells (Gibco, Life Technologies Corporation) containing 10% heat-inactivated FBS (Gibco, Life Technologies Corporation). Each cell was grown on 100-mm dishes incubated at 37 °C in 5% CO2.

**Confocal Laser Scanning Microscopic Images of Cellular Uptake.** A431 cells (4.7 × 10^4 cells/well, 200 μL) were plated onto an 8-well plate (μ-slide 8-well, ibidi GmbH, Gräfelfing, Germany) and incubated in MEM containing 10% FBS for 24 h at 37 °C under 5% CO2. After completely adhering, the cells were washed with MEM containing 10% FBS (170 μL/well). Before they were observed under a confocal microscope, the cells were stained with Hoechst 33342 dye (Thermo Fisher Scientific Inc.; 5 μg/mL) for 15 min at 37 °C. The cells were then washed with fresh cell culture medium and analyzed using an FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 40X objective without cell fixation.

**Flow Cytometry Analysis of the Membrane to Detect the Binding and Cellular Uptake of the Fluorescently Labeled Z33 Peptide and Dextran.** A431 cells (1.4 × 10^5 cells/well, 1 mL) were plated in a 24-well microplate (Iwaki, Tokyo, Japan) and incubated in MEM containing 10% FBS for 24 h at 37 °C in 5% CO2. After completely adhering to the wells, the cells were washed with MEM containing 10% FBS and treated with each sample (510 μL/well) for 24 h at 37 °C in 5% CO2 before being washed with PBS (triple washing, 200 μL), and subjected to the fluorescence intensity as measured by a Guava easyCyte (Merck Millipore) flow cytometer using 488 nm laser excitation and a 525 nm emission filter. In the case of trypsinization, after detachment of the cells, the cells were washed with PBS (400 μL) and centrifuged (800 g for 10 min). The cells were then added to PBS (200 μL/well) and subjected to fluorescence analysis with a Guava easyCyte flow cytometer using 488 nm laser excitation and a 525 nm emission filter. The cell viability was assessed using trypan blue staining.

**Immunostaining and Confocal Laser Scanning Microscopic Images (Anti-BSH Antibody Staining).** A431 cells (4.7 × 10^4 cells/well, 200 μL) were plated onto an 8-well plate (μ-slide 8-well, ibidi GmbH) and incubated in MEM containing 10% FBS for 24 h at 37 °C in 5% CO2. After completely adhering, the cells were washed with MEM containing 10% FBS (170 μL/well) at 37 °C for 10 min. The cells were then treated with 2 mM EDTA or 0.1 g/L-Trypsin/0.1 mmol/L/1-EDTA (200 μL/well) at 37 °C for 10 min. The cells were then stained using a FITC-labeled cetuximab (Iwaki, Tokyo, Japan) and observed using the Aperio Ultra confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 40X objective without cell fixation.
containing 10% FBS and treated with each Z33-BSH sample in MEM containing 10% FBS (170 μL/well) for 24 h at 37 °C. The cells were then fixed with 4% paraformaldehyde at 25 °C for 30 min and treated with 0.1% Triton X-100 in PBS at 25 °C for 15 min (200 μL/well). After washing with PBS, the cells were stained with anti-BSH antibody (A9H3 anti-BSH-Mab) in PBS (100 μL/well) for 1 h at 25 °C. Then, the cells were stained with Alexa-Flour 488 goat anti-mouse IgG (H + L) (100 μL/well) for 1 h at 25 °C. After washing with PBS, the cells were analyzed using an FV1200 confocal laser scanning microscope (Olympus) equipped with a 40× objective.

**ELISA.** A431 cells (1.0 × 10^6 cells/well) were plated onto a 60 mm cell culture dish for 24 h in 10% FBS-containing MEM. The medium was replaced with an equivalent medium containing each Z33-BSH sample in 10% FBS-containing MEM (900 μL). After incubating for 24 h at 37 °C under 5% CO₂, the cells were harvested with 0.05% trypsin/0.02% EDTA in PBS and suspended in total 2 mL of MEM. After cell counting, the cells were collected by centrifugation (370g) for 5 min. The pelleted cells were resuspended in 0.05% Tween-20 for 10 min. The boron concentration of the obtained cell lysate was determined by competitive ELISA using anti-BSH antibody A9H3 (N = 3).

**Cell Viability (WST-8 Assay).** Cell viability was analyzed using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] assay. A431 cells (1.4 × 10^5 cells/100 μL) were incubated in 96-well microplates in MEM containing 10% FBS for 24 h at 37 °C in 5% CO₂. The cells were then treated with each sample (50 μL) at 37 °C in 5% CO₂. After the sample treatment, the WST-8 reagent (10 μL) was added to each well, and the samples were incubated for 40 min at 37 °C. The absorbance at 450 nm (A450) and 620 nm (A620) was measured, and the viable cell number was calculated by subtracting the A620 value from the A450 value.

**Binding Assay of Z33-DB and Cetuximab.** Z33-DB (final 200 nM) and cetuximab (final 100 nM) were complexed in PBS buffer for 30 min at 25 °C, prior to ultrafiltration using Amicon Ultra-0.5 centrifugal filters (100,000 molecular weight cut-off, Merck) (10,000 g at 4 °C for 10 min) to remove nonbound Z33-DB to cetuximab. Bound Z33-DB concentration to cetuximab was detected using HPLC (Hitachi Chromaster and Chromassist data station).

**Thermal Neutron Irradiation and Assessment of the Cell-Killing Effect (Surviving Fraction).** A431 cells (2.0 × 10^5 cells/well, 2 mL) were plated into a 6-well microscope plate (Iwaki) and incubated in MEM containing 10% FBS for 14 days at 37 °C to examine colony formation. The colonies were counted with an incubator (Olympus) equipped with a 40× objective.

**Notes**

The authors declare no competing financial interest.
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